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INDUCTION AND COURSE OF PERMANENT DIABETES PRODUCED BY ANTERIOR PITUITARY EXTRACT

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THE OBSERVATION of Young (1) that permanent diabetes could be produced in dogs by the injection of anterior pituitary extract has been followed by numerous investigations. The present literature on this subject is fully cited in a recent paper by Marks and Young (2). Our initial report (3) on the production of diabetes in dogs by this method was based on the early results in 3 animals, and since that time 7 more dogs have been prepared. On the basis of a more adequate experience with these 10 animals further studies are here presented. These include additional observations on the induction of the permanent diabetes, and evidence for the pancreatic nature of the established diabetes based on the response to insulin, the fasting metabolism and the examination of the tissues. Also, the course of the permanent diabetes has been charted and the possible influence of insulin treatment on its progress has been noted. Studies on the respiratory metabolism, blood fats and the effect of dietary changes in these animals will be reported subsequently. From observations made during the past three years we have endeavored to select results which will supplement the rapidly growing knowledge of this type of experimental diabetes.

In this paper the term pituitary-diabetes will be used, according to the definition of Marks and Young (2), to describe the permanent diabetes established by a short period of pituitary treatment, although from present knowledge this diabetes is not associated with a continued overactivity of the pituitary gland.

The term total urinary diabetes will be used to describe the condition of animals which, during fasting, manifested the urinary glucose and nitrogen excretion of fasted depancreatized dogs. (See under results.)

METHODS

Animals. All of the dogs made permanently diabetic have been males with the exception of *P29*. The results of *P16*, *P19* and *P21* have been reported in part (3), under numbers 1, 2 and 3 respectively. Dogs *P26* and *P29* had part of the pancreas removed before they were made diabetic. Because the pancreatic remnants were large these dogs have resembled the other animals in every respect and hence are included in this series. The totally depancreatized animals used for comparison were prepared by the usual operation followed by the use of insulin until healing was complete, when insulin was withdrawn and the studies made.

The animals were kept in metabolism cages and the voided urine collected under toluene. As they were not catheterized, daily values have been calculated from consecutive 4-day periods. Occasionally these periods were 3 or 5 days, due to dietary changes. After permanent diabetes was established the animals usually ate well.

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Extract. The extract used was a crude saline extract of frozen and carefully dissected anterior lobes of beef pituitary glands. Its preparation has already been described (4). It was injected intraperitoneally, usually every day, during the course of treatment.

Diets. During injection all the animals (used for nitrogen studies) were fed raw beef heart, trimmed free of gross fat, and supplemented by bone ash, yeast, and 5 cc. of cod liver oil daily. *Dog P16* had cracker meal and fat added to the meat in its control periods and in some of the periods during injection. The amount of food offered during extract treatment was ample and uneaten portions were weighed to determine the daily intake. There was usually a slight decrease in the amount eaten during the first few days of the intraperitoneal injections, and an increased voluntary intake of food in the latter part of the treatment period. However, the amount of food given during control periods before extract treatment approximated the quantity of food during the periods of injection with which they are compared. Other details of the diets will be noted under results.

For the nitrogen content of the trimmed beef heart the average (2.75%) obtained by 5 duplicate analyses made at various periods has been used. The available glucose has been

TABLE 1. (DOG P16)

Diet	Total available glucose	Average glucose excreted	Average glucose utilized	Glucose utilized
gm.	gm.	%	%	gm.
1000 beef	108	81	19	20
700 beef	76	85	15	11
140 beef+60 sucrose	75	80	20	15
140 beef+180 sucrose[1]	195	81	19	37

[1] Isocaloric with 700 gm. beef.

calculated from the nitrogen content of the diet using a D:N ratio of 3.65. The glucose that might have been formed from the glycerol of the small amount of fat in the beef and from the glycogen has been calculated from an analysis of the fat and glycogen content and an arbitrary correction has been made. Eight grams of glucose of non-protein origin per 1000 gm. of trimmed beef heart was the correction used. In this way the available glucose from protein and the available glucose of the diet have been distinguished. In our previous paper (3) a slightly lower value for nitrogen was used and no allowance was made for the other sources of glucose detailed above.

The significance of the percentage of the available glucose excreted in the urine as an approximate measure of the severity of the diabetes is illustrated by the results obtained with different diets in *dog P16* (table 1). These data represent the averages of two or more 4-day periods. In the case of the diet of 1000 gm. of beef heart, 18 4-day periods were measured and the proportion of glucose excreted per period ranged from 72 to 89% with an average of 81%. Because this animal did not have total urinary diabetes (and presumably produced some insulin) there was an increase in the glucose utilized when more was available, an increase which fell within the range of the glucose equivalent of insulin.

The methods of chemical analysis have been the same as in our initial report (3). Tissues for liver and muscle glycogen were obtained under sodium pentobarbital anesthesia, the muscle being rapidly frozen in situ before removal. Liver and muscle glycogen were determined by a modification of the method of Cori, and liver fatty acids by the method of Stoddard and Drury.

RESULTS

Studies During the Induction of Pituitary Diabetes

Time and dose of extract required to produce persistent diabetes. The general treatment and behavior of all the animals has conformed to our early experience (3) and is in agreement with the detailed description of Young (5). The duration of pituitary

treatment and the quantity of anterior pituitary glands required in various dogs has been summarized in table 2. In the 6 dogs with intact pancreas that were given increasing doses of extract there was a large variation in the time required and in the total amount of extract needed to produce permanent diabetes. All of these animals ate well enough so that differences in food intake are not considered to be responsible for the individual variations. As the same lot of extract was at times used in different dogs with widely differing results, the variations noted cannot be explained solely on the basis of possible fluctuations in the potency of the extract.

Diabetes following the injection of small constant doses of extract. In 1934, Evans, Meyer, Simpson and Reichert (6) reported persistent glycosuria in 2 of 4 dogs injected

TABLE 2. ANTERIOR PITUITARY EXTRACT (APE) GIVEN TO PRODUCE PERMANENT DIABETES IN DOGS

Dog No.	Initial body weight	Period[1] of injection	APE[2]	
			Total	Average daily
	kg.	*days*	*gm./kg./period*	*gm./kg./day*
Dogs Given Increasingly Larger Doses				
P16	8.5	43	103.2	2.4
P19	5.7	33	70.4	2.1
P21	9.7	30	81.2	2.7
P22	8.7	24	35.6	1.5
P23	10.4	24	42.0	1.8
P27	4.9	14	26.6	1.9
Dogs Given Small Doses				
P30	19.4	62	16.8	0.3
P36	9.2	98	48.0	0.5
Partially Depancreatized Dogs				
P26	7.0	24	14.6	0.6
P29	12.9	27	15.5	0.6

[1] Injections were omitted for as many as 8 consecutive days within these periods.
[2] Expressed as grams of anterior pituitary gland from which the extract used was made.

for 8 and 9 months with a small constant dose of growth hormone. With this in mind two dogs, P30 and P36, were injected with small doses of pituitary extract. Table 2 shows that after 62 days *dog* P30 was found to be permanently diabetic although extract from only 16.8 gm. of anterior pituitary per kg. had been given. *Dog* P36 developed permanent diabetes after 98 days. In these dogs only occasional urinalyses were made until glucose was found after a month or more of injection. Omitting the extract for a few days resulted in the disappearance of the glycosuria. Resuming the injection of the usual small dose led to a return of glycosuria which progressed irregularly until the permanent diabetic state was obtained.

The effect of partial pancreatectomy on the time and amount of pituitary treatment. Table 2 also shows the influence of removing part of the pancreas in these animals.

Two animals, P26 and P29, failed to develop more than a trace of glycosuria during the injection of large amounts of pituitary extract (maximum of 25 gm. of gland per day), although they ate adequately. *Dog* P29 had been injected for 7 months with both small and large doses of extract without manifesting glycosuria, but she also gave no evidence of developing antihormones to the pituitary extract. This was grossly in-

dicated by the continuation of the discharge of a colustrum-like fluid from the breasts, a bloody vaginal discharge and her acromegalic appearance (7). At the end of this period insulin resistance was found, indicating that this component of the diabetogenic activity of the extract was still effective.

In both dogs about 1/5 of the pancreas was left intact so that they had no glycosuria during the 2 to 3 weeks of convalescence. They needed only 14.6 and 15.5 gm. of anterior pituitary per kg. to become diabetic in contrast to the minimum of 26.6 gm. in the intact dogs. Moreover, in the partially depancreatized animals the extract was administered in small daily doses which were not increased during the 24 and 27 days of injection.

The effect of pituitary extract on nitrogen excretion during the induction of diabetes. At various times during the injection of increasing doses of anterior pituitary extract

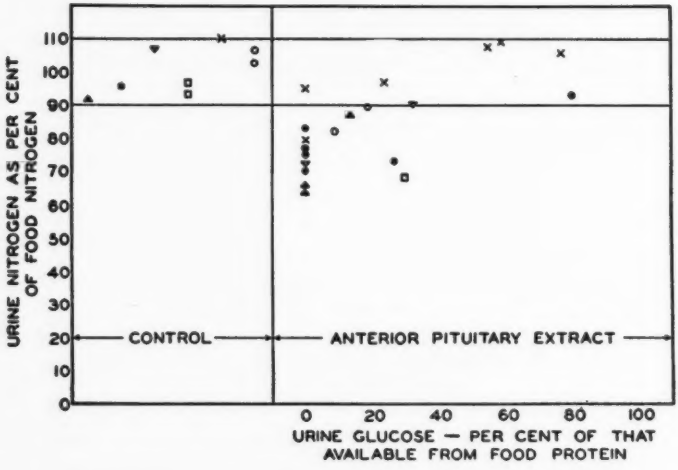

Fig. 1. Relation of urinary glucose and nitrogen excretion during injection of anterior pituitary extract. Each type of symbol represents an individual dog. The percentages charted were calculated from the data of 4-day periods.

the urinary nitrogen excretion was determined. This has been related to the dietary nitrogen and to the glucose excretion in figure 1, which summarizes the data on 6 animals before (control) and during the period of treatment. Urinary nitrogen has been expressed as a percentage of the average nitrogen content of the food. It is apparent that there was a distinct retention of nitrogen during those periods when there was little glycosuria attributable to protein sources. However, when the glucose from protein increased to about half of the quantity available from protein there was no longer nitrogen retention. As noted, there were some irregularities of food intake during injection but it was possible to obtain suitable periods for comparison.

Changes in weight are not graphically presented but, as would be expected, there was weight gain during the periods of nitrogen retention and loss of weight when the glycosuria represented more than half of that available from the protein in the diet. A linear relationship between changes in weight and in nitrogen excretion could be demonstrated when the percentage change in body weight per day was plotted

against the percentage of dietary nitrogen excreted and when the food eaten was appropriate to the weight of the individual animals.

Studies During the Course of Permanent Diabetes

The tendency of pituitary diabetes to progress in severity after the injections of extract have been discontinued has been noted by Young (5) and was observed in our first animals. The progress of the condition may be measured by the increasing glycosuria and ketonuria. In order to illustrate the course of the diabetes, its severity has been indicated by the proportion of the available glucose of the diet excreted in the urine. The curves in figure 2 have been prepared from the daily average of consecutive periods of 3 to 5 days.

In general, diets of 108 gm. of available glucose or less were composed of beef heart and diets higher in available glucose consisted of beef and sucrose. Dogs *P19* and *P21* also had cracker meal added to beef at times. All diets contained the vitamin and bone ash supplements.

The course of diabetes without insulin treatment. The data on 4 dogs (*P16*, *P19*, *P21* and *P22*) permitted the observation of the progress of the diabetes without insulin treatment. In figure 2 it may be seen that the diabetes of *P19*, *P21* and *P22* steadily increased until 85% or more of the available glucose of the diet was excreted. These animals attained this degree of diabetes in 40, 27 and 35 days, respectively, after the last injection of pituitary extract. In *dog P16* the diabetes did not become this severe for 152 days, but during this time there were many periods of little or no glycosuria due to fasting, fat feeding and also to anorexia and diarrhea during an attack of distemper. This interval is not included in the chart, but following recovery from distemper the proportion of the available glucose excreted began to increase rapidly and continued to do so after the glycosuria had again been interrupted by a period of high fat ingestion which ended on the 138th day. After the last point shown in figure 2 the severity of the diabetes in *P16* was such that about 80% of the available glucose was excreted. When the exacerbation of the diabetes had begun, in all 4 dogs, it continued fairly rapidly until 85% or more of the available dietary glucose was excreted. Young (2, 5) has also noted that the level of 85% glucose excretion occurred in several of his animals. Three of our animals progressed beyond this point and became as severely diabetic as depancreatized animals. (See below.)

The course of the diabetes with insulin treatment. The effect of insulin treatment upon an apparently rapidly progressing diabetes may be seen in the charts of dogs *P23* and *P30* (fig. 2). From the experiences described above in dogs without insulin it would be expected that these dogs would excrete 85% of the available glucose within 60 days following the lowest glycosuria. However, this was not the case in *P23*, in which insulin was started during a period of exacerbation which began like that of the untreated animals. With insulin treatment the diabetes of this dog did not progress at the rate of the untreated animals.

In *dog P30* the severity of the diabetes had reached 82% by the 129th day. As with *P23*, insulin was omitted at intervals to follow the course of the disease. The slope of the uncharted line joining the points indicating the periods when no insulin was given suggests that the advance of the disease was distinctly delayed by insulin therapy.

It seems, therefore, that in both insulin-treated animals the rate of progress of the diabetes has been retarded. It has been the impression of many clinicians that insulin may delay the progress of diabetes mellitus as well as improve the manifestations of the disease, but the accurate measurement of this in man is difficult.

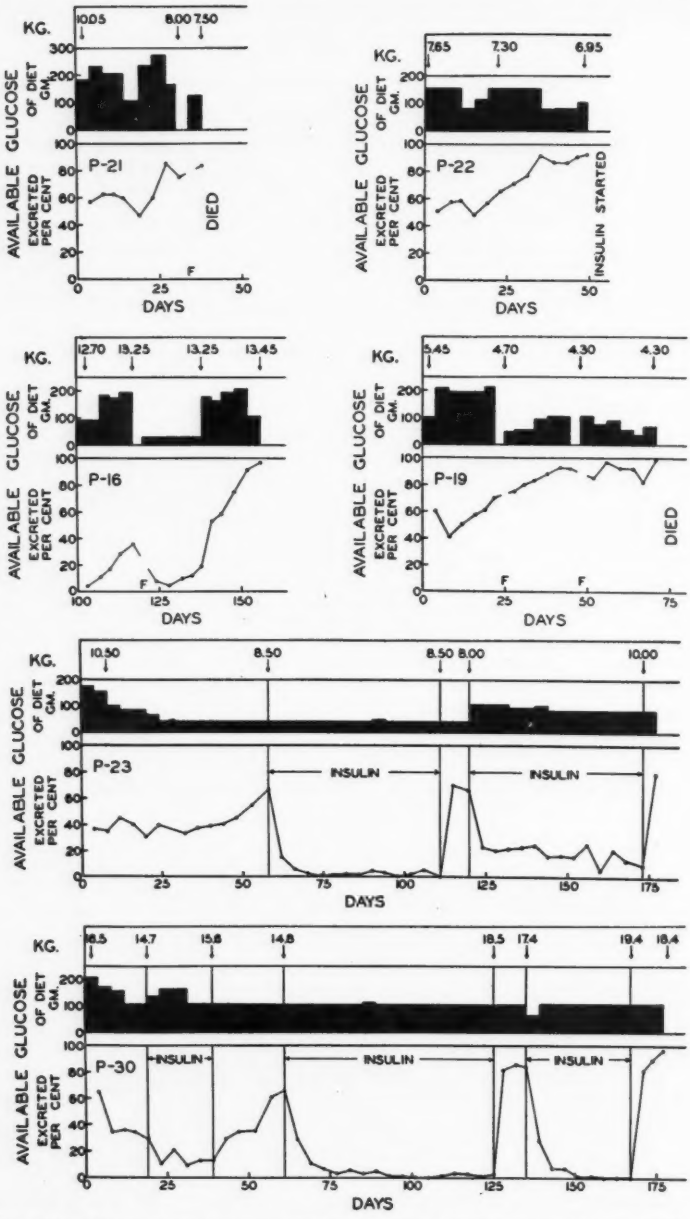

Fig. 2. THE PROGRESS OF THE DIABETES AFTER DISCONTINUING ANTERIOR PITUITARY EXTRACT. The solid black area represents the average daily available glucose of the diet.

The curve shows the percentage of this available glucose which was excreted in the urine. The daily average of consecutive 4-day periods has been plotted on the last day of each period (see text).

F indicates a fasting period. The days are numbered from the last injection of pituitary extract.

In connection with this beneficial influence of insulin it may be added that the injection of insulin without adequate control of the diabetes did not stop the progress of the condition in dog *P26* (not charted). At a time when it had no fasting glycosuria insulin was given for 25 days in such doses that an average of 25% of the available glucose was excreted and slight ketonuria developed. After this interval of inadequate treatment the fasting glycosuria of this dog was similar to that of the fasting depancreatized animal. From this single observation it seems that fairly complete control of the glycosuria is necessary if the course of the diabetes is to be favorably affected.

The possibility that the earlier use of insulin might have a restorative effect was tested in dogs *P29* and *P36*, in which insulin treatment was commenced 7 and 10 days, respectively, after the conclusion of pituitary extract when both dogs were ex-

TABLE 3. EFFECT OF FAT FEEDING OR FASTING UPON THE SUBSEQUENT EXCRETION OF GLUCOSE IN DOG *P16*

Days after APE[1]	Available glucose of diet excreted in urine				Duration of fat-feeding or fasting	Average fat ingested	Weight[3]	
	Before[2]		After[2]					
	Period 1	Period 2	Period 1	Period 2			Initial	Final
	%	%	%	%	*days*	*gm./day*	*kg.*	*kg.*
Mild Diabetes—Fat Feeding								
44	20	17	28	58	7	100	12.55	12.00
Severe Diabetes—Fat Feeding								
271	86	86	69	77	8	86	12.45	11.90
681	86	76	52	61	5	94	11.50	11.15
Severe Diabetes—Fasting								
149	91	97	72	72	7	—	13.45	12.30
294	78	87	74	79	7	—	12.30	11.40

[1] First day of first period.
[2] Two consecutive periods of four days before and after fat feeding and fasting.
[3] Weight at beginning and end of fat-feeding or fasting.

creting about 55% of the available glucose of their diet. Although there was a temporary decline in the insulin requirment of *P29* these animals failed to show any lasting improvement.

The influence of changes in regime on the course of the diabetes. a). When the diabetes was mild an increase in its severity occurred after stopping insulin abruptly. The diabetes has also become worse upon the resumption of a high carbohydrate diet after a period of pure fat feeding or after a day or more of fasting. Examples of the relation of such short fasting periods to the course of the diabetes are not shown in figure 2, where the average of 4-day periods is used. One animal (*P23*) had no such disturbance of regime associated with the exacerbation of its diabetes. Nevertheless, it is our impression that in animals not already severely diabetic an abrupt change to conditions increasing hyperglycemia may affect their course unfavorably.

b). When the diabetes was severe, that is, when it had reached a fairly level state with 80% or more of the available glucose excreted, the situation was altered. At this time the animal showed a transitory decrease in glycosuria following fasting or fat

feeding after which the diabetes returned to (but not beyond) its preceding severity. Table 3 illustrates the temporary effect of such dietary changes on the course of the diabetes in one dog when the condition was mild and later when it was severe. It is evident that the effect of dietary changes of this type and duration must be considered in relation to the severity and duration of the diabetes.

Miscellaneous observations during the course of diabetes. Because of the possibility that increasing the ingestion of specific amino acids of an organ might hasten the regeneration of that organ, *dog P22*, at a time when it showed total urinary diabetes, was fed 400 gm. of boiled pancreas and 400 gm. of beef heart daily for 7 weeks; *dog P16* received similar treatment when severely diabetic (85%). Insulin was given to both animals during this experiment and no evidence of physiological improvement was noted. Liver feeding, without insulin treatment, was also ineffective in *P16*.

Scott and Fisher (8) have reported that the zinc content of the pancreas of patients with diabetes mellitus is decreased. The concentration of zinc in the pancreas of one normal and four permanently diabetic dogs has kindly been determined for us by Dr. J. H. Austin. The values were as great in the diabetic dogs as in the normal dog. A modification of the dithizone method of Caughey, Holland and Ritchie (9) was employed. It is also of interest that zinc oxide in doses of 500 to 1600 mg. per day added to the diet, with or without insulin treatment, had no effect upon the severity of the diabetes of dogs *P16* and *P26*. This result might be expected when one considers the wide distribution of zinc.

Ophthalmoscopic examinations were made at irregular intervals in 9 of the dogs by Dr. Robb McDonald of the Department of Ophthalmology. Only one dog developed cataracts. This dog, *P22*, was noted to have normal lens, media and fundi at the beginning of the period of injection with anterior pituitary extract. On the 49th day, after the dog had become severely diabetic, some attenuation of the arteries of the fundi was noted. The lens and media were clear. Insulin administration was begun on the 50th day. On the 154th day bilateral immature cataracts showing many spoke-like opacities in the periphery were found. No fundus details could be distinguished. Between the 50th and the 154th days insulin had been omitted for 3 to 6 days on six separate occasions in order that the effect of fasting, fat feeding, and sucrose or meat diets might be noted. These periods were associated with moderate to severe ketonuria and a marked elevation of serum fatty acids and blood sugar. The diabetes as judged by urine glucose and nitrogen excretion was as severe as that of the completely depancreatized dog. On the 207th and 305th days, after further short periods of ketonuria and markedly elevated serum fatty acids, the cataracts were observed to be much more advanced with many opacities in the anterior and posterior cortex. Dogs are known to develop spontaneous cataract, but the rather rapid development during the period in which insulin was frequently omitted with the resultant total urinary diabetes is reminiscent of clinical observations. *Dog P23* with clinical and postmortem evidence of interstitial nephritis had no detectable ocular lesions though it must be realized that the brilliant tapetum of the dog may obscure minor pathological changes in the fundus.

The other dogs showed no ocular lesions. It is especially noteworthy that *dog P16* observed for a period of two years has not developed any permanent ocular lesions. Transitory lipemia retinalis was noted in *P16* and *P36* following fat ingestion.

X-ray examinations of the right hind legs of *dog P22* on the 221st day and of *dog P16* on the 423rd day were made by Dr. E. P. Pendergrass. These failed to show evidence of arteriosclerosis or bone lesions.

Complete blood counts and red blood cell sedimentation tests were made by Dr. A. J. Creskoff of the Department of Hematology. *Dog P16* on the 214th day had a

normal blood count and blood sedimentation rate, while dog *P22* on the 213th day showed a slight anemia and a moderately increased sedimentation rate. This animal had been subjected to frequent periods of ketosis as noted above.

The response to insulin and insulin requirement. The findings reported (3) on a single dog given a test dose of insulin by vein have been extended to a number of animals and the results are summarized in figure 3. All dogs were tested with 0.1 unit of insulin intravenously in the absence of severe acetonuria or infection. The absolute

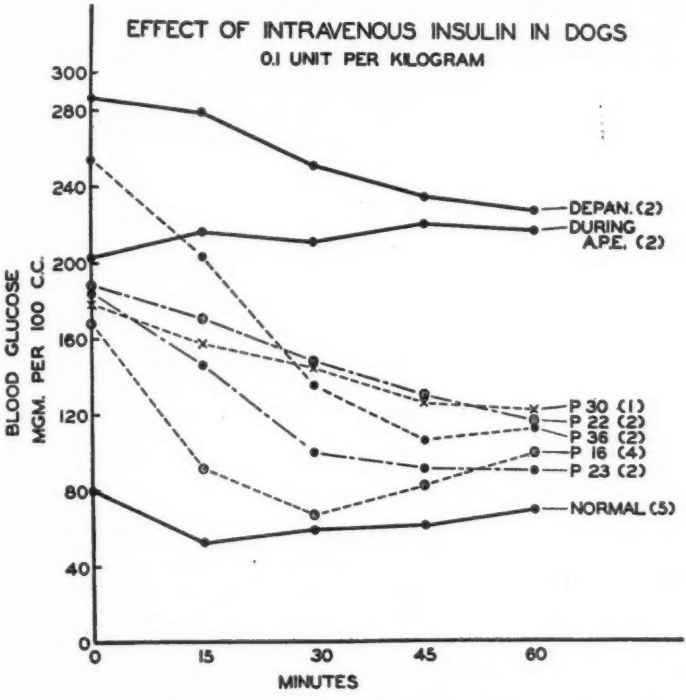

Fig. 3. The response to insulin. The broken lines show the average fall in blood sugar after insulin in each of 5 dogs which were permanently diabetic after anterior pituitary extract.

The solid lines show the average response to the same dose of insulin in 4 normal and 2 depancreatized dogs and in 2 dogs tested during the injection of anterior pituitary extract (APE).

The figures in parentheses indicate the number of tests.

decrease in blood sugar of the permanently diabetic dogs was somewhat greater than in the depancreatized dogs as was the maximum percentile fall in the glucose level. The drop in blood sugar exceeded that in normal animals, a finding which is not unexpected in the presence of an initially high blood sugar. The results gave no evidence of insulin resistance in the stage of permanent diabetes. They have been compared with the curves of dogs during the period of injection of anterior pituitary extract at which time there is no fall in the blood sugar following the same dose of insulin. Both dogs tested during extract treatment were selected for comparison because the initial blood glucose values were at the same general level as those of the permanently diabetic animals. The results agree with our initial tests and with the findings of Young

(5) and they afford evidence that the diabetes which persists after treatment with pituitary extract is not due to a continued or increased overactivity of the pituitary.

Marks and Young (2) have also presented evidence that in fed animals the amount of glucose retained per unit of insulin (the glucose equivalent) is greater in depancreatized than in pituitary-diabetic dogs. Campbell, Keenan and Best (19) have made similar observations. Direct comparisons have not been made between our pituitary-diabetic animals and depancreatized dogs on the same diets, but the glucose equivalent of insulin for 4-day periods in the 3 dogs with total urinary diabetes has shown a variability far in excess of any possible variations in the protein content of the beef diet. This is indicated in table 4. In dog *P22* the food was given 3 times daily in equal portions, and in *P26* and *P36* two equal portions were given. Insulin was given before each feeding.

It is clear that these animals follow the principle noted by Allan (11) that a high proportion of glucose to insulin favors a high glucose equivalent of insulin. However,

TABLE 4

Dog No.	Diet		Insulin	Range of glucose equivalent	No. of 4-day periods
	Meat	Sucrose			
	gm./day	gm./day	u/day		
P22	750	75	29	2.3– 5.1	10
P26	800	50	15	6.6– 8.0	5
P36	600	100	32	2.6– 4.0	3
	200 (a)	264	25	11.0–11.3	3
	1000 (b)	0	33	2.8– 3.0	4
	200 (c)	85	25	4.3	1

(a) and (b) are isocaloric; (b) and (c) are isoglucogenic.

the fluctuations of the glucose equivalent under constant conditions in our animals have been such that only extreme changes would be significant. The reasons for these variations are not apparent, although the presence of some endogenous insulin remains a possibility.

The degree of diabetes and its maximal severity. All investigators have observed that the severity of the permanent diabetes produced cannot be controlled or predicted during the period of extract treatment, and that after stopping extract the permanent diabetes may be of any degree of severity. When the tendency to progress is taken into consideration it is clear that in a group of animals the permanent diabetes will differ in its initial severity and that in a single animal its severity will vary at different times. During fasting in the milder stages there is little or no glycosuria and the nitrogen excretion is not increased above that of the normal fasted animal (3). However, in its maximal intensity, the diabetes may equal that of the depancreatized dog. This is illustrated in figure 4 in which the glucose and nitrogen excretion of our 3 most severely diabetic dogs during fasting is compared to that of depancreatized dogs.

Liver glycogen and fatty acids. It has been shown by Houssay (12) and others that during the first week of anterior pituitary extract injection a liver glycogen content above normal may be found in spite of a marked glycosuria. This is in contrast to the well known fact that untreated depancreatized dogs have a very low content of liver glycogen. Table 5 shows that the liver glycogen of our diabetic dogs has varied with the severity of the disease, previous insulin, and possibly with the type of diet, but values as low as those in depancreatized animals have been found. Except for *P36* the

liver fatty acids have not been markedly elevated under the conditions at the time of analysis.

In pituitary-diabetic dogs *P23*, *P30* and *P36* the muscle glycogen values varied from 0.44 to 0.66% whereas those of the 3 normal dogs ranged from 0.69 to 1.24%. In

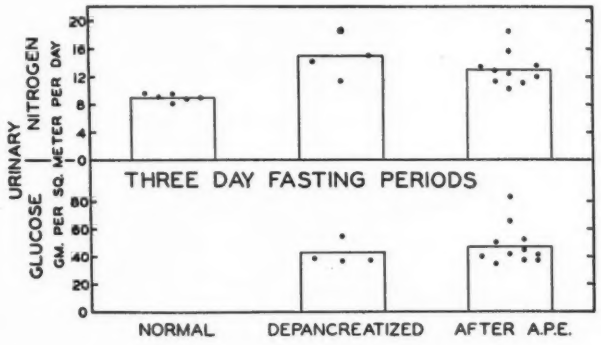

Fig. 4. AVERAGE DAILY URINARY GLUCOSE AND NITROGEN EXCRETION DURING THE FIRST 3 DAYS OF FASTING. Each point represents a fasting period. Four normal and 4 depancreatized dogs were used. Dogs *P22*, *P26* and *P36*, which became the most severely diabetic after stopping anterior pituitary extract (APE) were selected for this experiment. Surface Area (Sq. meter) = $0.112\ W^{2/3}$

the same pituitary-diabetic dogs the total alcohol-ether soluble material of the muscles varied from 6.2 to 7.1% and in 2 normal dogs values of 3.7 and 5.7% were found.

Autopsy data. At biopsy or autopsy the tissues were put in Zenker's bichromate solution without acetic acid for 8 hours. After washing the tissues were embedded in paraffin and sectioned. All tissues were stained with Delafield's hematoxylin and aqueous eosin. Sections of the pancreas were also stained by the Bensley-Mallory method to differentiate the alpha and beta cells of the islands. In a few instances other stains were tried (Azure II-eosin; Masson's stain), but they added nothing of importance. The pituitary glands were stained by Mallory's method.

TABLE 5

Dog No.	Severity[1] of diabetes	Time since last insulin	Time since removal of food	Type of diet	Liver	
					Glycogen	Fatty acids
	%	*hours*	*hours*		%	%
P23	78	42	42	Milk	2.96	2.09
P19	90	[2]	29	Meat and sugar	—	9.52
P21	90	[2]	24	Meat and sugar	2.96	6.85
P22	100	22	22	Meat and sugar	3.10	—
P26	100	29	29	Meat	0.59	6.15
P30	100	240	18	Meat	0.33	4.72
P36	100	214	22	Meat	0.16	16.35
Normal	—	—	26	Meat and starch	5.68	—
Normal	—	—	29	Meat	1.64	2.28
Normal	—	—	28	Meat	2.77	2.47

[1] The severity of the diabetes is expressed as the percentage of the available glucose of the diet excreted in the urine at a suitable period before the analyses of the liver.

[2] No insulin treatment.

TABLE 6

Dog	Duration[1] of glycosuria	Severity[2] of diabetes	Condition of islands				
			Number	Size	Hydropic degeneration	Atrophy	Bensley stain
	days	%					
Permanent Diabetes After Pituitary Treatment							
P19	102	90	Very few	Small	+	Marked	—
P21	68	90	1–2 seen	Small	Trace	Marked	—
P22	239	100	3–4 seen	Small	0	Marked	α cells only
	372	100	3–4 seen	Small	+	Marked	
	418	100	Very few	Small	Trace	Marked	
P23	236	78	Few	Small	0	Distinct	α and few β cells
P26	113	100	Very few	Small	0	Marked	α cells only
	158	100	Very few	Small	0	Marked	α cells only
P29	92	72	Few	Small	0	Marked	α cells only
P30	160	84	Slightly decreased	Small or normal	+	Distinct	
	202	100	Slightly decreased	Small or normal	0	Distinct	α cells only
P36	49	61	Very few	Small	0	Marked	α cells only
Temporary Diabetes During Pituitary Treatment							Terminal blood glucose mg. per 100 cc.
P8	5(8)[3]	Severe	Normal	Normal	+	Slight	555
P13	8(13)	Moderate	Normal	Normal	0	0	101
P17	7(11)	Slight	Normal	Normal	0	0	104
P31	2(3)	Slight	Normal	Normal	0	0	198
P24	0(7)	None	Normal	Normal	0	0	126
P14	17(26)	Severe	Normal	Normal	+++	0	506
P35	3(12)	Slight	Normal	Normal	0	0	88

[1] Total duration of glycosuria during and after the injection of pituitary extract.
[2] The severity of the diabetes is expressed as the percentage of the available glucose of the diet excreted in the urine during a period of several days preceding the biopsy or autopsy.
[3] Figures in parentheses show the number of days of pituitary treatment.

The pancreas. The animals studied were divided into two groups; *a*), dogs which were permanently diabetic after pituitary treatment, and *b*), dogs which had temporary diabetes during the injection of anterior pituitary extract. The latter group was composed largely of animals which responded to the extract with only a slight glycosuria. Table 6 summarizes the findings in both groups and relates them to the degree and duration of the diabetes.

a). *In permanently diabetic dogs* the pancreas has been examined only after fairly

long periods of glycosuria. Following partial pancreatectomy or biopsy the usual moderate scarring was found at the site of operation. Otherwise all pancreatic tissue was grossly normal and no instances of pancreatitis as described by Richardson (13) were encountered. In these animals the early changes in the islands such as hydropic degeneration were slight and atrophy of the islands was the characteristic lesion (fig. 5 and 6). In some dogs only one or two small distorted islands per section were found; such atrophy was not accompanied by fibrosis. Such findings recall Allen's (14) description of the islands in prolonged diabetes after partial pancreatectomy: "As the islands shrink by loss of degenerated cells, the acinar tissue closes in smoothly about them.—There is no connective tissue reaction and not even a leucocyte comes to devour the debris." Other dogs showed varying degrees of atrophy indicated by

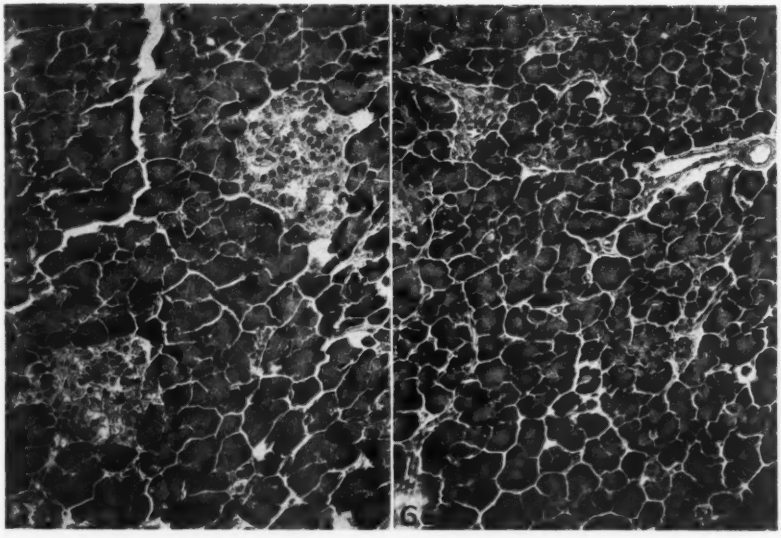

Fig. 5. Islands of normal dog. Fig. 6. Islands of diabetic *Dog P23*—same magnification. The photographs were made by Mrs. Miriam R. Barrett from the Laboratory of Pathology.

the decrease in the number of islands and a diminution in their size due to loss of beta cells. The differential stain of the islands usually showed that they were composed largely of alpha cells and these were diminished in number in many instances. One cannot exclude the presence of a few beta cells even in the most severely diabetic animals but the phrase 'alpha cells only' used in table 6 conveys the general impression given by these sections. The relation of the lesions to the severity of the diabetes was highly approximate but suggestive. On the other hand it was not possible in these experiments to observe any influence of insulin treatment on the lesions. Our results are in accord not only with Allen's (14) report but also with the observations of Richardson (13) and Campbell, Keenan and Best (10) in pituitary-diabetes studied at comparable periods.

We have not seen hyaline degeneration of the islands which was observed by Richardson (13) in 3 of his dogs, 2 of which had pancreatitis and the third "slight inflammation of the intralobular connective tissue." The possible relationship of hya-

line degeneration and pancreatitis noted by Richardson (13) may explain our failure to observe hyaline change.

Four animals in our series showed slight vacuolation of the small intralobular ducts (13).

b). *Temporary diabetes in dogs during the injection of pituitary extract* has been investigated by us only incidentally and our sections have not permitted the study of degranulation of the beta cells described by others (13, 15). The results in 7 such animals are shown in table 6. All but 2 (*P8* and *P14*) failed to respond with an adequate glycosuria so that they were observed in the refractory state. Hence, they do not illustrate the progress of island lesions in animals destined to become diabetic. Dogs *P8* and *P14* with marked hyperglycemia at the time of autopsy showed distinct hydropic degeneration. The third animal with hyperglycemia (*P31*) had only been injected for 3 days, a period too brief to permit the development of this lesion (14). The remaining animals of this series showed no abnormalities of the islands and this result is associated with the absence of severe diabetes at the time of examination. Hydropic degeneration of the epithelium of the small ducts which has been described by others (13, 15) was noted in only 2 dogs (*P17* and *P31*). In general the results in this series of animals are in agreement with those of Allen (14), Richardson (13) and Ham and Haist (15) for dogs made diabetic by partial pancreatectomy or pituitary extract for similar periods.

The thyroid glands of the permanently diabetic animals (*group a*) appeared within normal limits in every instance. However, in the animals examined during pituitary treatment (*group b*) the thyroid uniformly presented marked hyperplasia indicative of the thyrotropic action of the crude extracts used.

The adrenal glands manifested no consistent abnormality. In one permanently diabetic dog with nephritis (*P23*) and in 2 of the animals from *group b* the combined weight of the adrenals exceeded the maximum weight for normal dogs reported by Baker (16). Otherwise the adrenals were within normal size and no distinct microscopic evidence of hyperplasia was found. Mitotic figures, which have been described by Ham and Haist (15), were not observed in the hyperplastic thyroids or in the adrenal cortex, but very few of our animals were studied at the time most appropriate for observing them.

The pituitary, heart, lungs and blood vessels have shown no definite abnormalities. *Dog P36* at autopsy showed moderate fatty infiltration of the liver. In the livers of the other dogs there has been only slight fat deposition, not regarded as significant. *Dog P23* had interstitial nephritis and *P36* had slight fibrosis of the kidney. In the remaining animals the kidneys were normal. *Dog P36* was autopsied after 267 days of glycosuria, at which time the pancreas appeared the same as it did at the 49th day (table 6). The duration of observation of the other animals is shown in table 6 by the maximum period of glycosuria. *Dog P16* has had no examination of the pancreas and is still living, 2½ years since it became diabetic.

DISCUSSION

The observations made during the period when pituitary extract was administered show the dosage of extract and the duration of treatment and include *a*), animals made diabetic by small doses of extract, and *b*) animals which could not be made diabetic until part of the pancreas was removed. It has been our experience that permanent damage of the pancreatic islands required a minimum time of 14 days for its production and that this interval is but little decreased by partial pancreatectomy. On the other hand, much less pituitary extract is needed to produce the metabolic condi-

tions under which diabetes develops if the island tissue has been reduced by partial pancreatectomy. The same principle appears to apply to the differences between species. Young (4) could not produce glycosuria in normal rats, but after partial pancreatectomy Long (17) found this species susceptible to diabetogenic substances. Similar results have been obtained in the cat (4, 18). Hence both individual and species differences in the temporary or permanent response to agents causing glycosuria appear to be in large part the reflection of differences in pancreatic function.

In the case of the resistance to pituitary extracts, which may be developed in some animals after a prolonged period of treatment, an additional factor must be considered. It has been shown (19) that under certain circumstances antihormone effects, obtained by the prolonged administration of pituitary extract, may modify pancreatic diabetes. As these effects were observed in the absence of the pancreas it is unlikely that antihormones played any great part in the refractory state of dogs P26 and P29 in which their resistance to the extract was overcome by removing part of the pancreas. The rôles played by pancreatic function and by antihormone phenomena can only be estimated as their coexistence is always possible.

At the present time gain in weight (20), nitrogen retention as indicated by studies of the blood (21) or urine (22), glycosuria and the insulin content of the pancreas (23) are some of the measurements used to study the interrelationship of the growth and diabetogenic activities of the pituitary on the one hand, or of growth and insulin function on the other hand. The discussions of Mirsky (21) and of Young (20) survey the recent literature. Gaebler and Zimmerman (24) have subsequently reported findings in phlorhizinized dogs which are related to the results in pancreatic and pituitary diabetes. They showed that growth hormone caused some nitrogen retention and gain in weight in phlorhizinized bitches and that glucose excretion was proportionately diminished so that the D:N ratio remained unchanged. In contrast they noted the diabetogenic effect of their extract in totally depancreatized dogs and they postulated an increased output of insulin in normal and phlorhizinized animals to account for the lack of diabetogenic effect in these animals. This assumption is supported by the finding of Cori (25) that the insulin content of the pancreas of phlorhizinized cats is only slightly below normal. Although the reduced insulin content of the pancreas during pituitary treatment (23) cannot be related directly to the rate of insulin secretion, it appears that the progressive failure of insulin production would best explain the failure of our animals to retain nitrogen when glycosuria was severe. Our results are in harmony with the conception that if sufficient insulin is available pituitary extract may induce nitrogen retention; but that if the available insulin is decreased, the same extract may be diabetogenic.

Although the course of the diabetes after the cessation of pituitary extract has been briefly noted (5), it seemed to merit a more detailed description. There are many obvious differences between the conditions under which diabetes rapidly develops during pituitary treatment and the conditions under which it slowly progresses after pituitary extract has been withdrawn, and it is clear that the whole course of the disease must be kept in mind when any aspect thereof is under study.

In the case of dietary changes we have noted certain differences in the response of the animals depending upon the state of the diabetes at the time the observations were made. Although these results are suggestive they are less striking than those obtained with the use of insulin.

The investigations on insulin in the whole course of pituitary-diabetes may be summarized as follows.

1. During the injection of pituitary extract the normal response to a test dose of insulin is abolished (pituitary glycotropic effect).

2. The use of large doses of protamine insulin daily during the injection of pituitary extract prevents the development of diabetes, of hydropic degeneration of the islands and of the reduction in the insulin content of the pancreas which follow pituitary treatment without insulin (23).

3. In the permanent phase there is a normal response to a test dose of insulin.

4. In the permanent phase, treatment with insulin retards the progress of the diabetes. The insulin requirement in some dogs (2) may be greater than that of depancreatized dogs.

5. In dogs in which atrophy is the island lesion, no restoration of the islands or recovery of the animal has followed insulin treatment of the established diabetes.

6. In cats (18), during the period when the lesion of permanent diabetes is hydropic degeneration, treatment with insulin may lead to morphological restoration of the islands accompanied by lasting recovery of the animal after insulin is withdrawn.

These investigations are all in harmony with the concept that the permanent phase of pituitary diabetes is the result of damage to the pancreas. Finally, in addition to the response of these animals to diet and insulin, certain well-established signs of pituitary activity may be mentioned. Hyperplasia of the thyroid was noted during the injection of extract (thyrotropic effect) and this was not present in the permanent phase of diabetes. Examination of the few female dogs injected, in these and other experiments, showed that the gonadotropic effect was exerted by our extracts and that it disappeared when extract was stopped. Ham and Haist (15) noticed mitotic figures as evidence of adrenotropic activity during the injection of pituitary extract, but we have not happened to observe this phenomenon in our animals. Although the varied activities of the pituitary may not all rise and fall together, the facts indicate that the permanent phase of diabetes is not due to the continued overactivity of the pituitary.

SUMMARY

Observations have been made upon the induction period, course and nature of the permanent diabetes that occurred in 10 dogs after the intraperitoneal injections of anterior pituitary extract.

The induction period. 1. Six normal dogs, given increasingly larger doses of anterior pituitary extract, became permanently diabetic after variable amounts of extract had been given over periods of 14 to 43 days.

2. Two normal dogs failed to develop more than slight glycosuria during the injection of large doses of anterior pituitary extract. Partial pancreatectomy without resultant glycosuria was followed by permanent diabetes in both dogs after a small amount of extract had been administered.

3. Two normal dogs given small, fairly constant doses of extract became permanently diabetic after 62 and 98 days of treatment.

4. Nitrogen retention was found during the injection period when the glycosuria attributable to protein sources was not severe.

The course of the diabetes. 1. In 4 dogs, which were not treated with insulin during the first few months of diabetes, the proportion of the available glucose of the diet excreted in the urine increased progressively until it amounted to 85 per cent or more.

2. In 2 dogs treated with insulin the rate of progress of the diabetes seemed to be inhibited.

3. The effect of changes in the regime of the animals on the progress of the disease appeared to be related to the severity of the diabetes at the time such changes were made. In one dog, excreting about 80 per cent of the available glucose of the diet,

periods of fasting or fat feeding were followed by a temporary diminution in the severity of the diabetes.

The nature of the diabetes. The following evidence agrees with previous work in supporting the concept that the diabetes which continues *after* stopping anterior pituitary extract is primarily due to loss of function of the islands of Langerhans.

1. In the pancreas of the 8 dogs examined, atrophy of the islands of Langerhans was conspicuous and the remaining islands consisted chiefly of alpha cells.

2. In the most severely diabetic dogs the response to insulin, the fasting urinary glucose and nitrogen excertion and the liver glycogen were similar to the findings in totally depancreatized dogs. In addition, manifestations of the thyrotropic and gonadotropic as well as of the glycotropic (contrainsulin) effects of pituitary extract observed during the period of injection were absent in the permanent phase of diabetes, suggesting that overactivity of the pituitary was not responsible for the persistent diabetes.

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THE HEPATIC GLYCOGEN RESERVES IN DIABETES MELLITUS[1]

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IN THE CLINICAL EVALUATION of the patient with diabetes mellitus it should be of interest not only to determine the patient's insulin requirement, but also to establish the clinical severity of the disease. Of the various methods hitherto suggested to determine the latter, the most satisfactory would appear to be the rate at which the diabetic patient develops ketosis and coma when subjected to the appropriate insult. However, since this cannot be predicted, it is impossible to determine which patient will develop coma during an infection and which patient will develop only a moderate ketosis.

In the attempt to devise some procedure for gauging the degree of diabetic 'severity' it became apparent that the clinical goal is a measure of the susceptibility to diabetic coma. Since the latter is in large measure dependent upon severe ketosis, it is apparent that a measure of the development of acetonemia will also serve as a gauge of susceptibility to coma.

There is ample evidence that acetone bodies are normal products of fatty acid oxidation in the liver and that they appear in excessive amounts in the blood stream only when their rate of production exceeds their rate of oxidation by the muscles (1, 2). Furthermore, it is also acknowledged that, with few exceptions, the stimulus for fatty acid oxidation and consequently for acetone body production is a depletion of liver glycogen (3).

It is evident that the rapidity with which a liver may be depleted of glycogen is dependent to a great degree upon the amount of glycogen present in that organ. Hence a measure of the ability of the liver to maintain its glycogen should serve as an index of the rapidity with which acetone body formation may occur. Thus, a determination of hepatic glycogen 'reserve' would serve as a gauge of the patient's susceptibility to ketosis and coma, which in turn, could be regarded as an index of the clinical severity of the diabetic patient. Toward this end, an attempt has been made to devise a procedure which would permit the determination of the glycogen 'reserves' of the liver.

Since Claude Bernard (4) first enunciated the principle that the liver has a glycogenic function and serves to keep the circulation supplied with sugar, it has been established that the liver is the only source of endogenous glucose (5). Thus, it is generally accepted that the blood sugar level is the resultant of a dynamic balance between the glucose secreted by the liver into the circulation and that removed for

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utilization by the tissues. Therefore, any deviation from the normal blood sugar level must be due to a disturbance of one or the other aspect of the balance (fig. 1).

It is obvious that the ability of the liver to maintain an adequate secretion of sugar is dependent in large measure upon its glycogen content. In the normal organism in which the hepatic glycogen concentration is relatively high, a sudden increase in the removal of sugar from the blood stream should produce little change in the blood sugar level so long as glycogen is available in the liver. However, if glycogen store of the liver is low, a similar removal of sugar from the blood stream should result in a fall of the blood sugar to a level where gluconeogenesis provides a new supply of sugar for the blood stream. Furthermore, if the liver has a low glycogen content and uses this glycogen to compensate for a loss from the blood, it should become further impoverished and consequently will draw on fat and protein for energy, with the

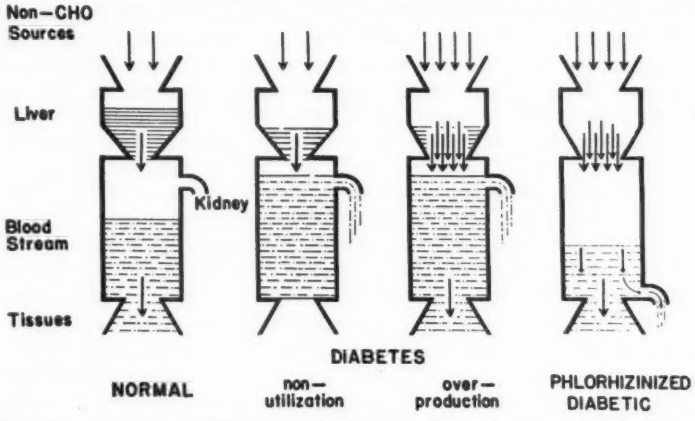

Fig. 1. Schematic representation of the relationship between the carbohydrate of the liver, blood stream, and tissues, illustrating (*a*) dynamic balance between the secretion of glucose by the liver and its utilization by the tissues of the normal organism, (*b*) the disturbance occurring in diabetes mellitus, and (*c*) the influence of phlorhizination.

secretion of acetone bodies as one of its products. The appearance of a significant acetonemia would indicate definitely that the liver glycogen had reached a minimal level.

On the basis of these considerations, a procedure has been developed whereby it may become possible to gauge in man the capacity of the liver to maintain a normal concentration of glycogen. By lowering the renal threshold for sugar by means of phlorhizin administration, a sudden and rapid drain of the sugar from the blood stream occurs. This results in a compensatory increase of hepatic glycogenolysis in an attempt of the liver to maintain the blood sugar level, thereby depleting its glycogen reserves (fig. 1). The loss of sugar via the kidneys can be measured in the urine excreted during the experimental period. As long as the blood sugar level remains unchanged, the excreted glucose must represent glucose secreted by the liver; that is liver glycogen. However, when there is a decrease in the level of the blood sugar, the glucose excreted in the urine represents not only glucose which has been secreted by the liver, but also some free sugar which was originally present in the blood and other body fluids. By appropriate calculations the sugar which must have originated in the liver can be estimated. Furthermore, if an acetonemia occurs concomitantly with the development of hypoglycemia, it becomes evident that the liver has had only minimal amounts of glycogen and that the sugar in the urine now takes origin from newly formed glucose in the liver.

It is apparent that if the liver of the diabetic organism cannot retain normal amounts of glycogen, the administration of phlorhizin with the consequent decrease in the renal reabsorption of glucose will result in a lowering of the blood sugar level, while the blood of a normal subject will show no change after losing the same amount of sugar (fig. 1). The more severe the metabolic disturbance, the greater will be the loss of glycogen and hence the greater the rapidity with which a decrease in the blood sugar level will occur. Hence the ease with which hypoglycemia and acetonemia develop in consequence of glucose removal from the body via the kidneys provides a means for gauging the glycogen 'reserves' of the liver of the diabetic organism.

METHOD

After conducting a number of preliminary studies the following procedure was adopted. All patients, normal as well as diabetic, were permitted to have their regular evening meal on the day before the test. At 9 P.M. additional food was provided in the form of orange juice and cane sugar in an amount sufficient to furnish 2 gm. of carbohydrate per kg. of body weight. This extra carbohydrate was provided in order that every patient might have adequate carbohydrate for storage as hepatic glycogen. The next morning breakfast was omitted and no food was permitted during the test. The diabetic subjects did not receive their morning dose of insulin. Those diabetics who were receiving protamine insulin, were given regular insulin the day before the test. The test was begun at 7:30 A.M. and completed 6 hours later. Water was allowed ad libitum. A blood sample was drawn at the beginning and again at the end of the 6-hour period, and was analyzed for its sugar content by the Somogyi, Shaffer-Hartmann method,[4] and for total acetone bodies by a modification of the Van Slyke and Fitz method. Immediately after the withdrawal of the first blood sample, 1 gm. of phlorhizin dissolved in 0.1 N sodium hydroxide was injected intravenously. The subject then emptied his bladder. Since the effect of the injected phlorhizin was lost fairly rapidly (complete inhibition of glucose reabsorption lasting less than 3 hours), it was found necessary to administer another gram of phlorhizin intravenously at the end of 3 hours in order to maintain a constant glycosuria. The urine was collected during the 6-hour interval and the total amount of glucose excreted determined. At the end of the test period, the patient was permitted to eat in order to compensate for the loss of sugar from the body.

The preparation of the phlorhizin for injection consisted in the solution of 1 gm. of phlorhizin in 20 cc. of 0.1 N NaOH which had been boiled and permitted to return to body temperature. Numerous tests always revealed the solution of phlorhizin to be sterile. The solution was injected intravenously over a period of 3 to 4 minutes. In more than 175 subjects thus treated, no damage to the veins ever occurred.

RESULTS

The present report is based on a study of 48 adults and 40 children who were treated as described above. The diabetic subjects were with few exceptions under

[4] Using Reagent 50 and the aid of acid zinc sodium hydroxide method of deproteinization, we found that although recovery of added sugar was adequate, the absolute values were low by amounts relatively insignificant in macro determinations, but of decided importance in micro determinations. We found traces of zinc remaining in the filtrate to be the responsible factor for these discrepancies. On communicating with Dr. M. Somogyi, we learned that he had recognized this source of error and suggested a modification of his deproteinization technic (7). This procedure employs approximately 0.3 N barium hydroxide, so adjusted that 5 cc. are required to titrate 5 cc. of 5% zinc sulphate solution alkaline to phenophthalein. For 1:10 filtrates, 1 volume of blood is laked with 5 volumes of water and 2 volumes of barium hydroxide added and mixed. Two volumes of zinc sulphate are then added and the mixture centrifuged and filtered. For micro filtrates; 0.2 cc. blood are rinsed into 7 cc. water and to this 0.4 cc. barium hydroxide and 0.4 cc. zinc sulphate are added separately. The mixture is centrifuged and a 5 cc. aliquot is withdrawn into a cotton tipped pipette. Using this method of deproteinization and the Somogyi micro reagent we have secured excellent results.

good clinical control and their insulin requirements varied from zero to 120 U per day. The diabetic patients receiving no insulin were maintained aglycosuric by dietary restriction.

It is evident from table 1 and figure 2 that the average change in the blood sugar of normal adults consequent to phlorhizin administration is relatively insignificant and within the limits of experimental error. In the diabetic adults (table 2) a marked drop in the blood sugar occurs. Thus, whereas in the normal group the blood sugar is decreased by an average of 3%, a decrease of 48% occurs in the diabetic group (fig. 2).

TABLE 1. THE INFLUENCE OF PHLORHIZIN IN NORMAL ADULTS

No.	Age	Sex	Weight	Initial blood sugar	Fall in blood sugar	Glucose excreted in urine	Total decrease in free sugar	Loss from liver	Rise in blood ketones
			kg.	*mg. %*	*mg. %*	*gm.*	*mg./kg.*[1]	*mg./kg.*	*mg. %*
16	24	M	82.0	55	+11	34.6	+33	455	0
39	32	M	76.3	74	1	23.3	3	302	0
2	32	M	76.3	87	+14	24.2	+42	359	0
7	45	M	45.4	65	+15	10.9	+45	285	0
8	56	M	71.0	81	1	17.3	3	241	0
9	41	F	51.0	58	+1	12.5	+3	248	0
14	63	F		99	9	17.4			0
15	50	F		66	0	17.7			0
81	29	F		84	5	17.0			0
94	60	M	45.4	88	6	14.0	18	290	0
68	44	M	74.6	81	7	23.0	21	287	0
32	60	M	66.0	75	4	14.0	12	200	0
33	60	M	66.0	74	+1	22.3	3	341	0
27	19	M		49	7	15.0			0
34	36	M		66	4	21.9			0
37	49	M		65	10	20.4			0
38	33	M		62	18	21.7			0
44	37	M		90	4	26.5			0
46	40	M		99	9				0
50	26	M		82	0	28.0			0
51	27	M		76	5	25.0			0
150	38	M	70.0	88	2	6.4	6	85	0
91	37	F	57.7	87	10	15.5	30	239	0
41	26	F	49.5	61	1	22.0	3	437	0
40	25	F		78	6	19.9			0

[1] mg./kg. body weight.

In contrast to a 3% reduction in the blood sugar level of normal adults, an average decrease of 12% is produced by phlorhizination of normal children (table 3, fig. 2). The diabetic children all show a marked decrease in blood sugar (table 4) which is greater than that observed in the diabetic adults (fig. 2). It is evident from the data that the diabetic subject invariably has a decrease in the blood sugar concentration consequent to phlorhizination. Of interest is the fact that although the majority of the patients were under good clinical control so far as their diabetic status was concerned, nevertheless, marked differences were noted as compared with the nondiabetic group.

The glucose excretion varied markedly from pateint to patient and from group to group (tables 1–4). Examination of the data reveals that not only do differences in body weight play a role in the variations of sugar excretion, but that age and clinical status are important factors. In view of the many variables which influence the amount of sugar excreted in consequence of the phlorhizination, an attempt to compare the groups on the basis of total glucose excreted yields little information. However, the quantity of glucose excreted becomes of importance in the estimation of the amount of glycogen lost by the liver during the experimental period. The computation of this amount is based upon the following considerations. The glucose in the

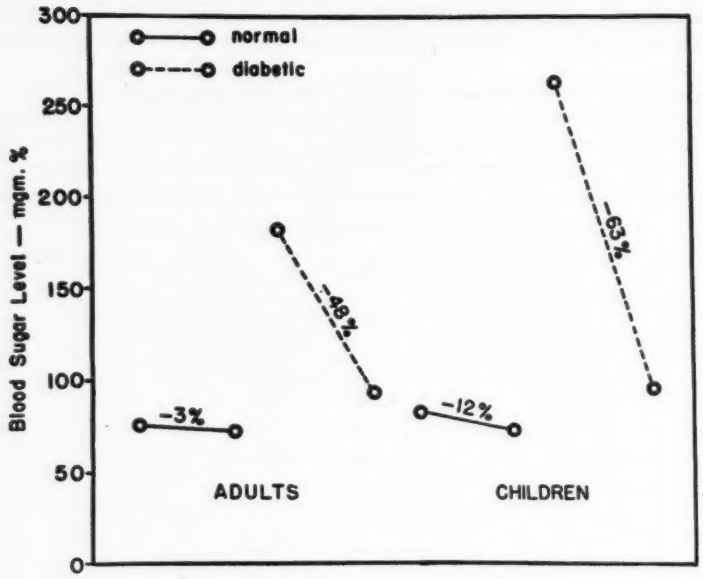

Fig. 2. THE INFLUENCE OF PHLORHIZIN ON THE BLOOD SUGAR LEVEL OF NORMAL AND DIABETIC ADULTS AND CHILDREN.

TABLE 2. THE INFLUENCE OF PHLORHIZIN IN DIABETIC ADULTS

No.	Age	Sex	Weight	Insulin dosage	Initial blood sugar	Fall in blood sugar	Glucose excreted in urine	Total decrease in free sugar	Loss from liver	Rise in blood ketones
			kg.		mg. %	mg. %	gm.	mg./kg.	mg./kg.	mg. %
69	60	M	66.4	0	151	29	28.0	87	335	0
17	60	M	62.7	50[1]	42	3	9.6	9	144	0
18	72	M	45.4	30[1]	162	115	18.5	345	63	17.3
21	63	M	44.5	20[1]	104	52	21.2	156	323	12.6
19	17	M		75[1]	210	77	42.5			2.6
24	52	M		15[1]	203	108	33.7			3.1
31	47	M		0	204	77	43.4			0
3	68	M		0	259	159	22.4			13.4
10	62	M	72.0	30[2]	169	102	14.5	306		0
73	28	F	43.2	0	89	29	16.0	87	283	10.5
1	47	F	60.0	5[1]	84	29		87		15.5
67	53	M	48.6	10[2]	338	202	52.0	606	464	23.5
123	24	M	52.3	0	93	9	15.8	27	275	0
124	61	M	55.5	0	231	94	39.5	282	430	0
76	45	F	78.2	0	141	33	25.0	99	221	0
36	22	F	47.2	30[1]	194	108	24.9	324	204	6.3
63	62	F	71.1	30[2]	282	112	21.0	336	41	0
61	52	F	45.5	30[1]	155	47	23.9	141	385	0
43	51	F	52.3	40[1] 10[2]	261	161	37.3	483	230	13.7
45	50	F	56.8	0	270	171	54.0	513	437	9.8
28	17	F		40[1]	121	79	22.0			7.1
53	51	F		20[1]	214	117	29.0			0
54	52	F		0	218	110	28.0			0

[1] Protamine.
[2] Regular.

urine is excreted from the blood stream, and the sugar of the blood is in turn secreted by the liver. In order to estimate the proportion of the excreted sugar originating from the liver glycogen, it is necessary to take into account the total change in the blood sugar. When the blood sugar level remains constant, all of the urinary sugar must have come from the liver. On the other hand, if there has been a fall in the blood sugar level, then the amount of sugar excreted in the urine is equal to the sum of that secreted by the liver plus the sugar equivalent to the decrease in the blood and tissues.

In order to make the above estimations, it becomes essential to determine the significance of a change in the blood sugar in terms of the body as a whole. Various values for mixing volume for sugar distribution in the body are found in the literature.

TABLE 3. THE INFLUENCE OF PHLORHIZIN IN NORMAL CHILDREN

No.	Age	Sex	Weight	Initial blood sugar	Fall in blood sugar	Glucose excreted in urine	Total decrease in free sugar	Loss from liver	Rise in blood ketones
			kg.	mg. %	mg. %	gm.	mg./kg.	mg./kg.	mg. %
57	13	M	52.3	84	5	21.0	15	386	0
60	8	M	25.0	86	11	14.0	33	527	0
65	11	M	33.6	85	2	15.0	6	440	0
98	5	M	19.1	84	6	9.6	18	484	7.0
99	10	M	32.5	87	8	13.2	24	382	0
100	5	M	20.9	90	23	11.3	69	471	8.0
103	13	M	39.6	90	3	18.5	9	458	2.2
105	14	M	46.8	81	11	15.6	33	304	0
106	13	M	51.4	85	17	23.6	51	408	□
48	12	M	38.6	81	+5	15.8	15	425	□
92	4	M	14.7	82	23	15.1	69	958	13.3
114	5	M	16.4	87	18	10.4	54	580	7.4
56	12	F	42.3	73	□	19.0	□	449	0
89	11	F	33.3	91	3	18.7	9	553	0
96	12	F	45.9	85	7	27.1	21	569	0
97	16	F	50.4	91	9	21.1	27	392	0
101	8	F	25.5	95	20	16.4	60	582	6.1
107	14	F	53.4	70	+2	20.6	6	392	□
108	13	F	57.8	73	2	16.3	6	276	3.0
109	11	F	30.0	82	18	12.6	54	366	3.0
110	12	F	32.5	88	15	19.8	45	564	0
112	8	F	21.1	76	22	9.3	66	375	10.6
113	9	F	28.9	82	15	11.8	45	363	□
117	9	F	23.4	90	11	13.4	33	539	0
118	11	F	34.2	82	8	16.4	24	456	0

These vary from 10 to 50% of the total body weight. Of these, a value of 30% has been assumed to approximate the true mixing volume.[5] Using this distribution factor it was possible to compute a change in blood sugar in terms of mg. of glucose per kg. of body weight. Thus, for example, *case* 2, whose weight was 76.3 kg,. showed a rise in the blood sugar of 14 mg.%, at the end of the 6-hour interval, corresponding to an increase of 42 mg. of free sugar per kg. of body weight. This patient excreted 24 gm. of sugar during the experimental period, i.e., 317 mg. per kg. of body weight. In view of the fact that the liver was responsible for the addition of 42 mg. of glucose per kg. of body weight, the total loss from the liver was 42+317 mg. of sugar per kg. of body weight. On the other hand, subject 82, weighing 38.6 kg., excreted 37 gm. of glucose and showed a decrease in the blood sugar of 273 mg.%. Thus 958 mg. of sugar per kg. of body weight was excreted in the urine. Of this amount, 819 mg. per kg. of body

[5] Calculating our data with factors greater than 30% for mixing volume, we occasionally obtained figures which are beyond possibility. If the latter be disregarded, the use of a factor greater than 30% tends to reduce the "loss of sugar from the liver" of the diabetic subjects and would indicate still smaller 'reserves' than by the use of the 30% factor.

weight came from the free sugar of the blood and tissues. Hence the excretion of sugar by the liver (i.e., from hepatic glycogen) was 958−819 or 139 mg. of sugar per kg. of body weight.

Such computations permit a comparison of different cases within the groups and of one group with the other on the basis of the amount of extra glucose which was

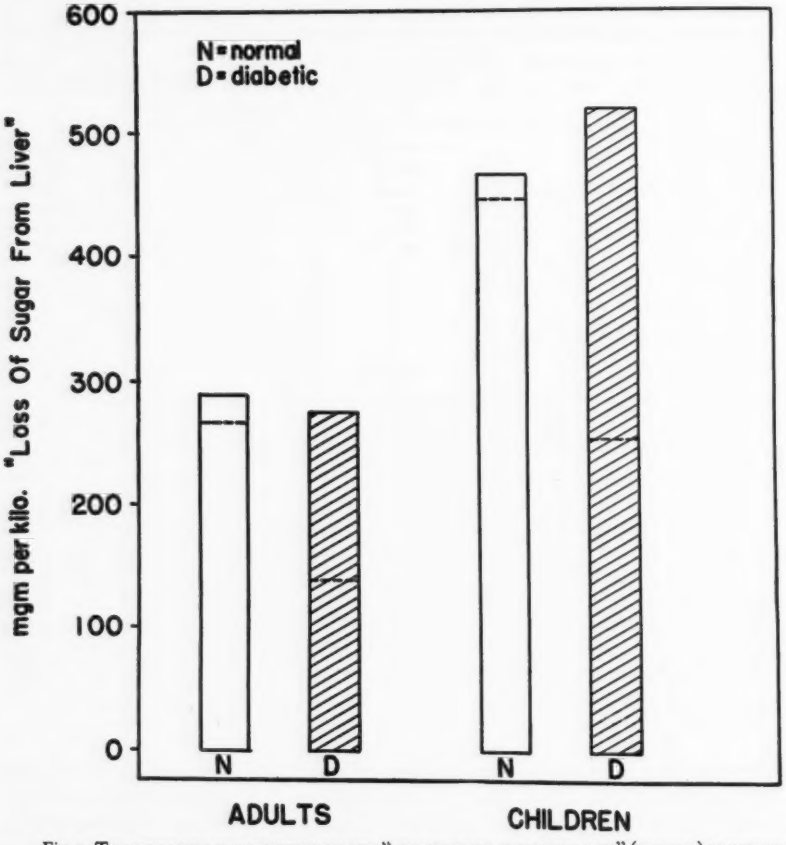

Fig. 3. THE INFLUENCE OF PHLORHIZIN ON THE "LOSS OF SUGAR FROM THE LIVER" (SEE TEXT) IN NORMAL AND DIABETIC ADULTS AND CHILDREN, CONSEQUENT TO PHLORHIZIN ADMINISTRATION. The cross-hatched line in the bars represents the data calculated on the basis of a 50% distribution of free sugar in the body, whereas the total bars refer to a calculation based on a 30% distribution. (See footnote—page 363.)

secreted by the liver during the experimental period. Thus the normal adults have an average loss of 289 mg. glucose per kg. of body weight during the experimental period (table 1, fig. 3), while the diabetic adults have an average loss of 274 mg. per kg. of body weight[6] (table 2, fig. 3).

[6] It is of interest to note with reference to footnote 5 that if a distribution factor of 50% is employed instead of 30%, the "loss from liver" of normal adults becomes 266 mg. per kg., while that from diabetic adults becomes 139 mg. per kg. emphasizing the low liver glycogen reserves of the latter. In children, the use of the 50% factor results in a "loss from the liver" of 449 mg. kg. from the normals and 252 mg. per kg. from the diabetics.

TABLE 4. THE INFLUENCE OF PHLORHIZIN IN DIABETIC CHILDREN

No.	Age	Sex	Weight	Insulin dosage	Initial blood sugar	Fall in blood sugar	Glucose excreted in urine	Total decrease in free sugar	Loss from liver	Rise in blood ketones
			kg.		mg. %	mg. %	gm.	mg./kg.	mg./kg.	mg. %
72	16	M	51.4	20[1]	155	101	23.0	303	144	3.5
75	6	F	23.6	25[1] 8[2]	167	127	11.0	381	85	10.7
82	10	F	38.6	25[1] 3[2]	375	273	37.0	819	139	3.3
84	12	F	38.4	75[1]	246	187	39.0	561	455	6.4
52	16	M	54.5	45[1] 30[2]	305	155	63.0	465	691	0
58	7	M	28.6	20[2]	448	192	52.0	576	1242	15.0
62	15	M	47.7	43[1] 25[2]	318	249	48.0	747	260	0
70	11	M	32.7	25[1] 10[2]	305	178	53.0	534	1085	7.3
102	16	M	47.8	65[1] 12[2]	234	175	29.6	525	94	0
119	11	M	31.8	27[1] 12[2]	366	245	48.1	735	777	7.3
55	15	F	42.3	37[1] 17[2]	154	87	29.0	261	425	0
59	10	F	31.8	22[1] 12[2]	248	149	35.0	447	653	11.5
64	12	F	39.5	30[1] 15[2]	361	233	74.0	699	1175	19.0
93	6	F	28.1	25[1] 8[2]	91	45	10.9	135	253	3.7
49	14	F	51.0	80[1] 40[2]	166	97	24.2	291	182	0

[1] Protamine.
[2] Regular.

Of interest is the fact that normal children show a proportionately greater loss of glucose from the liver than do adults, viz., an average of 468 mg. per kg. of body weight (fig. 3). The diabetic children likewise lose a large amount of glucose (from the liver, viz., an average of 510 mg. per kg. of body weight (fig. 3).

It is important to emphasize that the figure designated in tables 1–4 as "loss of sugar from the liver" does not represent the total amount of glycogenolysis, but is merely the compensatory additional breakdown consequent to the increased excretion of sugar by the kidneys. Although both normal and diabetic adult groups secrete approximately the same amount of extra sugar from the liver during the experimental period, only the normal group is able to maintain the original blood sugar level, while the diabetic group shows a decided drop in the blood sugar concentration. The obvious implication of this observation is that there was not enough hepatic glycogen present in the diabetic patients to permit adequate maintenance of their high blood sugar level.

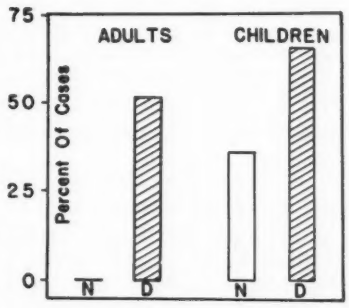

Fig. 4. INCIDENCE OF KETOGENESIS IN NORMAL AND DIABETIC ADULTS AND CHILDREN CONSEQUENT TO PHLORHIZIN ADMINISTRATION (N = NORMAL, D = DIABETIC)

It is noteworthy that the rate of extra glycogenolysis consequent to the increased glycuresis in normal children is very rapid as compared with that of normal adults. Thus the liver of the normal child secreted approximately 175 mg. of sugar per kg. more than did that of the normal adult during the 6-hour period. The diabetic children likewise secreted large amounts of extra sugar from the liver, demonstrating the ease with which it is possible to accelerate glycogenolysis in the child.

The data concerning the accumulation of acetone bodies in the blood are detailed in tables 1–4. Since ketosis will develop when the concentration of hepatic glycogen reaches some critical minimum (6), it is of interest to analyze the data on the basis of

the percentage of cases which do, or do not develop ketosis. Thus none of the normal adults (table 1) developed an acetonemia even though they lost as much hepatic glycogen as did the diabetics. With a loss from the liver of 289 mg. of sugar per kg. of body weight, the normal adults developed no ketonemia, while 52% of the diabetic adults developed a ketonemia on losing 274 mg. of sugar per kg. of body weight (fig. 4).

The development of acetonemia occurs with greater ease in the normal children as shown by the fact that 36% of this group develop an acetonemia at the end of the test period. This effect may be associated with the greater glycogenolytic rate noted above. Similarly, the diabetic children show a somewhat greater incidence of ketosis than do the diabetic adults; 66.6% in the former, as compared with 52% in the latter (fig. 4).

In summarizing the above data it may be stated that the removal of approximately the same amounts of glycogen from the liver results in an insignificant change in the blood sugar of normal subjects, in contrast to a marked depletion of the blood sugar of diabetic subjects. Furthermore, there is produced a greater incidence of acetonemia in diabetic patients than in either normal adults or children.

DISCUSSION

Although phlorhizin has been employed extensively to produce experimental glycosuria ('phlorhizin diabetes') it has never been shown to exert a toxic action unless administered for long periods of time to fasting animals (8). The use of phlorhizin in man and lower species indicates that the glucoside per se produces no organic changes. H. W. Smith and his coworkers have employed phlorhizin in their studies of kidney function in man with no apparent permanent injury. In 1933, Chasis, Jolliffe and Smith (9) summarized 25 reports on the use of phlorhizin in man and stated that "in no instance was permanent injury described." In another study, we have administered phlorhizin to a male adult for a period of 4 months, during which time the patient received 1 gm. of phlorhizin subcutaneously thrice daily. Repeated urea clearance determinations and urinary examinations revealed no evidences of impairment in kidney function. Hence we believe that the administration of phlorhizin, as outlined above, is not a deleterious procedure. It is important to note that phlorhizin is relatively ineffective in the production of glycosuria in patients with impaired renal filtration.

In evaluating the significance of these data, it is necessary to emphasize that the extra glucose excreted by normal subjects during the test period in consequence of phlorhizination is not due to gluconeogenesis. This is exemplified by an experiment in which the hourly excretion of nitrogen was studied before and after the injection of phlorhizin. No change whatsoever occurred in the rate of nitrogen excretion during the 6-hour period in which glucose was excreted and hence gluconeogenesis cannot be held responsible for the extra sugar excreted by the kidneys.

The fact that with approximately the same loss of sugar from the liver, the adult diabetic group shows a ketosis, while the normal group does not, suggests that the glycogen reserves of the diabetic were inadequate at the outset of the experimental period, since otherwise the blood sugar level would have remained constant. The only effect of phlorhizin is that of any other glycogenolytic agent. Thus, the administration of epinephrin may produce a greater rise in the blood sugar of normal subjects than in that of diabetics (10), indicating a poverty of hepatic glycogen in the latter. However, phlorhizination provides a more efficient procedure for measuring the amount of sugar lost by the liver. It permits a measure of the capacity of the liver to retain carbohydrate in amounts sufficient to prevent ketogenesis and indicates the

course of events which may ensue, consequent to the development of an infection or any other glycogenolytic stimulus.

It is generally acknowledged that children are more susceptible to the development of diabetic acidosis than are adults. Quantitative evidence substantiating this clinical impression is provided by the greater ease with which both normal and diabetic children developed acetonemia consequent to the depletion of their glycogen stores in the liver.

In view of the fact that with a given loss of extra sugar from the body, the diabetic subject develops a drop in the blood sugar level and an acetonemia, it becomes possible to categorize individual diabetics on the basis of their response to phlorhizination. At this point, it is of interest to note that patients suffering from hepatic dysfunction frequently have an impoverishment of liver glycogen. Hence it is not surprising to find that the results in such patients are similar to those obtained in diabetic subjects.

In a number of adult diabetic patients blood samples were drawn at frequent intervals and the presence of acetonemia determined, noting at the same time the quantity of glucose excreted. In this manner it was possible to determine the smallest loss of sugar from the body necessary to induce acetonemia. In many instances acetonemia developed when only 5 to 10 gm. of glucose were excreted, which indicated the poor glycogen reserves present in these patients. This approach provides another procedure for determining the glycogen reserves in diabetic patients.

It is obvious that the greater the decrease of the blood sugar level and the increase of acetonemia, and the smaller the amount of glucose that must be removed to induce these effects, the more severe must be the metabolic disturbance in the diabetic subject. Individual cases fit into this hypothesis, but a definite formula which will take all factors into consideration must await further experience.

SUMMARY

The administration of phlorhizin and the consequent glycosuria results in removal of circulating sugar. In conditions where the glycogen reserve of the liver is adequate the blood sugar remains relatively constant. In conditions where the glycogen reserve is inadequate the blood sugar level drops and ketosis may develop.

Diabetic adults do not retain carbohydrate in their livers to the same degree as do normal adults, and the removal of relatively small amounts of sugar from their blood stream results in further deprivation of liver glycogen with a consequent development of hypoglycemia and ketosis.

Children cannot retain carbohydrate to the same degree as do adults because of an apparent increased rate of glycogenolysis. This fact is true not only for diabetic children, but also for normal children and accounts for their greater susceptibility to ketosis and coma.

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AGENTS PROMOTING GASTROINTESTINAL ABSORPTION OF INSULIN

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SINCE THE INTRODUCTION of insulin into therapeutics periodic attempts have been made to produce insulin products which would be effective by mouth. Something like 16 different agents have been reported to be effective in animals. Very few of these have been effective clinically and none has proved satisfactory for therapeutic use. If success is to be achieved, the factors determining gastrointestinal absorption should be better understood. Recent attempts to produce an oral absorbable insulin indicate that more than inhibition of enzymic destruction of insulin is necessary. The breakdown of some physiological barrier in the intestine, such as selective absorption, in addition to enzymic inhibition, might produce the desired result. Or, some physiological carrier might be found for the insulin. This seemed all the more plausible in view of the known fact that insulin is not destroyed in the liver, because once it gets into the portal circulation it is just as active as when given into the systemic circulation (11).

It appeared possible to approach the problem pharmacodynamically, that is, by testing for effects on insulin action of agents with known properties and actions. Confirmation or refutation of the claims of others also appeared desirable. The net result was the discovery of a number of fairly simple agents, which can produce the desired result and one in particular in man. Although this agent is not satisfactory for general therapeutic use, it was felt that the data obtained should be recorded at this time as they might be valuable to others, and also because they appear to confirm suggested principles which control the gastrointestinal absorption of insulin.

It may be stated briefly that the following agents have been found by others to promote gastrointestinal absorption of insulin as indicated generally by hypoglycemia in normal animals, though rarely convulsions: 20% alcohol (1); blood serum or water (per rectum) (2); saponin (3); decholin, 60% alcohol, gelatin with calomel, 5% alcohol with cayenne pepper and glycerol (4); pinacol (tetramethyl glycol), also effective in pancreatectomized dogs (5); iron salts (6); hexylresorcinol and some other resorcinols, thiamin chloride, and sodium hexametaphosphate (7); aerosol (8). Generally, the successful results have been obtained in dogs and rabbits, sometimes mice and rats. The following agents have given negative results in animals: incubation with feces (9); bile and bile compounds (10); lecithin (11); trypan red (4); yeast, digitonin, strophanthin, ernutin, and acetylsalicylic acid (12). In human diabetes, the following are reported to have given positive, though fleeting and rather variable, results after oral administration: olive oil (13); mixture of saponin, trypan red and malachite green (14); 0.1 to 0.2% hydrochloric acid or 5 to 8% alcohol by duodenal tube (15); hexylresorcinol (normal men) (7). A mixture of cocoa butter, lactic and palmitic acids and saponin, given as a rectal suppository, is claimed to be effective in animals and diabetic patients (16). Bile given with insulin failed to promote absorption in children (17), which is in agreement with the failure of this agent in animals. Some of these positive and negative results have been confirmed by us, but the total number of agents used in our work represents a greater variety and scope.

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METHODS

The criteria of insulin absorption used were hypoglycemia and convulsions in animals, and hypoglycemia in man. The total number of different animals used was as follows: 191 rabbits, 100 mice, 6 guinea pigs and 7 rats. Several normal persons were used for a number of selected agents. Crystalline zinc-free insulin, crystalline zinc insulin and amorphous insulin[1] were used interchangably without demonstrable differences in action. The various agents were mixed directly with insulin before using. In some cases, the insulin was in solution, but in others, in suspension, according to the nature of the agent or vehicle.

In rabbits, the insulin mixtures were put directly into ligated loops of jejunum or ileum of 10 cm. length. The animals were anesthetized lightly with ether for a quick laparotomy for exposure of the intestine. After introduction of the insulin mixtures, the rabbits were released into cages. Injections into the colon or rectum were made with a blunt needle in unanesthetized rabbits. In mice, rats and guinea pigs, the insulin mixtures were introduced directly into the stomach with the aid of a suitable metal stomach tube. In man, the insulin mixtures were taken by mouth in gelatin capsules, or by rectum in gelatin capsules or cocoa butter suppositories. The dosage of insulin varied to some extent in all species. The usual doses were: rabbits, 200 U; mice, 5 U; rats, 100 U; guinea pigs, 25 U; and man, 500 U. Blood sugar was estimated by Folin and Malmros' method (18) at the time insulin was administered and again 1 to 3 hours afterwards. The results obtained have been grouped according to general or physical-chemical properties. The following results were obtained in animals, changes in blood sugar being averages and the number of animals being variable, but generally at least 2 for each result and up to 20 to 30.

Acids. The acid-insulin mixtures tried in intestinal loops reduced the blood sugar as follows: 0.2 cc. 5% hydrochloric acid, 50%; 0.1 gm. boric acid, 7%. There were no convulsions. These moderate effects could be due to inhibition of tryptic digestion, and agreed with the results of others in man (15).

Astringents. The 3 astringents tried in intestinal loops were tannin, 10%, ferric chloride, 0.1%, and zinc acetate 1 cc. of saturated solution. The tannin reduced the blood sugar 11%, the iron, 30%, without convulsions, and the zinc, 30%, with convulsions. Thus the absorption of insulin was only moderately promoted, despite the known general propermeability effects of astringency on cell surfaces, and the positive claims for iron.

Alcohols. (*a*) *Simple alcohols.* The alcohols tried in intestinal loops caused the following reductions in blood sugar, and symptoms: 1 cc. 10% alcohol no effect, 1 cc. 95% alcohol, 62%, with convulsions; 1 cc. propyl alcohol, 20%, without convulsions; 0.05 cc. benzyl alcohol, 37%, with convulsions. These results generally confirmed the known promoting effects of alcohol on enteral absorption of insulin, although the hypoglycemia was not marked.

(*c*) *Glycols.* Pinacol, 1 cc., in intestinal loops, reduced the blood sugar about 55% and caused convulsions; 1 cc. per rectum reduced the sugar 70%, with convulsions. Given gastrically in doses of 0.02 cc. to white mice, pinacol reduced the blood sugar about 25%, without convulsions. These results confirmed those of Major as to hypoglycemia in dogs receiving pinacol (5).

Propylene glycol, 0.1 to 0.6 cc. in the intestinal loops of 10 rabbits, reduced the blood sugar an average of 23%, with occasional convulsions. In 20 mice, the gastric administration of 0.02 cc. reduced the blood sugar an average of 14%, with convulsions. Accordingly, the promotion of enteral absorption of insulin is not specific for pinacol.

[1] All the insulin was supplied gratuitously by Eli Lilly & Co., Indianapolis, Indiana.

(c) *Alcohol-amines.* In intestinal loops, 2 cc. of 50% triethanolamine reduced blood sugar 50% and caused convulsions. Triisopropanolamine, in from 0.02 to 0.2 cc. in intestinal loops, reduced the blood sugar 23% and caused marked convulsions. Gastric administration of 0.5 cc. in rats reduced the blood sugar only 5%, without convulsions.

Mixtures of triisopropanolamine, 0.02 to 1.3 cc. and propylene glycol, 0.1 to 0.6 cc., in intestinal loops reduced the blood sugar 45% and caused convulsions. Mixtures of triisopropanolamine, 0.6 cc., and propylene glycol, 0.1 cc., per rectum in rabbits reduced the blood sugar 63% and caused convulsions. Accordingly, the results with these agents together appeared to be additive, the 2 together being much more effective for promoting absorption than either one alone.

Both of these agents enter into the composition of sobisminol solution and mass (N.N.R.), and contain an enteral absorbable bismuth compound. It was thought that this agent might be more efficient in promoting absorption than its glycol and amine ingredients, but 0.25 cc. of sobisminol solution in intestinal loops reduced the blood sugar 30%, with convulsions. The same dose per rectum in rabbits reduced the blood sugar only 11%, without convulsions. Gastric doses of 0.1 cc. in mice reduced the blood sugar only 12%, some with convulsions, and 0.05 to 0.1 cc. in guinea pigs not at all. Thus the bismuth complex in sobisminol failed to act as an efficient carrier of insulin.

Anisotonic solutions. Distilled water, in from 0.2 to 0.5 cc. in intestinal loops, raised the blood sugar 12%; 0.5 cc. per rectum in rabbits decreased it 21%, without convulsions. Gastric administration of 0.1 cc. in mice reduced the blood sugar only 5%. Five cc. of 15% sodium chloride solution in intestinal loops reduced the blood sugar 36%, without convulsions.

Dyes. According to Lasch and Schönbrunner (4, 19), acid or electronegative dyes protect insulin against inactivation by pepsin in vitro, and basic or electropositive dyes against inactivation by trypsin. These authors claim to have demonstrated such protection for insulin against peptic action by congo red and trypan red, both being acid dyes, and against tryptic action, by malachite green, a basic dye. They used members from both classes of dyes mixed with insulin in order to obtain a dual protection with single oral administration against the gastrointestinal enzymes. However, it was necessary to add saponin to the dye-insulin mixtures (tablets) in order to obtain practical results in human diabetes. It is rather surprising that this dye-saponin-insulin mixture should have been used, since saponin alone was equally effective in animals and man, and the dyes alone were ineffective for reducing blood sugar in animals.

However, we found that 0.1 gm. trypan red alone in intestinal loops reduced blood sugar 20%, and 0.1 gm. malachite green, 49%, with convulsions. A mixture of these dyes, in the same doses in intestinal loops, reduced blood sugar 27%. The following dyes in similar doses used similarly also reduced the blood sugar, namely, methylene blue 44%, acriflavine 54%, mercurochrome 16%, crystal violet 47%, gentian violet 58%, and thionol 49%. Convulsions occurred after methylene blue and crystal violet. Obviously quite a variety of dyes, unrelated chemically, and some are crystalloids and others colloids, can promote the enteral absorption of insulin. Practically all those tried above are electropositive (basic), and therefore, might prevent tryptic destruction of insulin according to Lasch and Schönbrunner (18). Whether this protective action is related to their electrical properties, or to a chemical antiseptic action, which all possess, is not known.

Irritant volatile oils. Simple hyperemia of the intestine might promote insulin absorption, but this would not necessarily be important if the absorption is selective.

Actually, 0.02 cc. mustard oil in intestinal loops, reduced the blood sugar 42% and caused marked convulsions. However, application of mustard oil to the exterior of the loops containing the same amount of insulin and a generalized hyperemia of the organ raised the blood sugar 12%. Peppermint oil, 0.05 cc. in intestinal loops, raised the blood sugar 5%. An entirely different type of volatile irritant, namely, 1 cc. of ammonium hydroxide, applied to the exterior of loops also raised the blood sugar, i.e., 10%. A number of agents described in previous sections also acted as local irritants; for instance, the alcohols, acids, amines, and others. However, those possessing lipoid solubility or cell surface effects, or both, were more consistent and efficient, and the volatile oils are also lipoid soluble. Therefore, it seems that simple hyperemia is not important in itself for enteral absorption of insulin, but some other property or action is necessary. This was borne out by results with different agents.

Nerve depressants. Some nerve depressants tried acted specifically on the motor, sensory and ganglionic elements, but others acted generally. All could be regarded as protoplasmic poisons. They were used in intestinal loops. Reductions in blood sugar were caused by the following: 2 cc. of 10% cocaine, 64%; 0.1 gm. ethyl aminobenzoate, 22%; 1 to 5 mg. atropin, 25%; 0.2 gm. chloral hydrate, 61%; and 0.1 gm. sodium cinchophen, only 5%. Convulsions occurred only after cocaine and chloral. Saligenin, 2 cc. of 10%, increased blood sugar 4%, sodium barbiturate, 0.2 gm., 15%, and nicotine sulfate, 1 to 5 gm., 18%. Accordingly, the majority of the nerve (protoplasmic) poisons tried were either ineffective or not outstanding promoters of insulin absorption. Only cocaine and chloral were definitely effective and these drugs are known for their depression of protoplasm. Furthermore, protoplasmic paralysis or death is known to favor cellular permeability and the favorable results on insulin absorption with cocaine and chloral were consistent with their local effects. Such a correlation of effects was supported by the striking favorable results with another powerful protoplasmic poison, namely, sodium fluoride, i.e., 0.1 gm. in loops reduced the blood sugar 37%, with marked convulsions. Although the protoplasm of normal living cells resists permeability, surface effects without paralysis or death of protoplasm, may be produced which favor it and might promote insulin absorption. This is indicated by the outstanding results with the agents described in the next section.

Quinine and related agents. The following salts of quinine, in 0.1 gm. doses in intestinal loops, produced the following marked reductions in blood sugar: quinine sulfate 52%, and 36% (per rectum), quinine tannate 71% and quinine hydrochloride 52%. Related alkaloids, under the same conditions, were less effective, the reductions in blood sugar being as follows: cinchonidine, 29%; cinchonine, 20%; hydrocupreine, 35% and ethylhydrocupreine, 34%. Convulsions occurred after quinine sulfate and hydrochloride and ethylhydrocupreine. Plasmochin and atebrine were ineffective, the blood sugar having been increased 1 and 18%.

A check was made on the antienzymic action of quinine as this might be a factor in the promotion of enteral absorption of insulin. Insulin and quinine were incubated with pepsin and hydrochloric acid, and with pancreatin and bicarbonate, the control mixtures containing all these ingredients, except quinine. Then all the mixtures were injected into intestinal loops in some rabbits, and subcutaneously in others. The controls produced no effects whatever, as might be expected. The mixtures containing quinine produced variable results as to blood sugar and symptoms, although, in general, the inactivation of insulin was less complete in the presence of quinine, thus indicating that a part at least, of the quinine effect was anti-enzymic.

Although quinine has an antienzymic action in vitro and in the alimentary canal, this action was not responsible for the fairly marked enteral absorption of insulin

from the ligated intestinal loops. Loops washed practically free from enzymes, as well as unwashed loops, reacted alike under quinine. Therefore, some other local effect must have been exerted by the quinine such as a cell-surface tension lowering action, or an inhibition of protoplasmic action, perhaps in mutual relationship. Certainly a marked poisoning comparable to that of the cocaine or fluoride used need not be postulated for quinine. That a surface tension lowering effect is important for enteral absorption of insulin is indicated by the following results with a number of different agents of this general type.

Surface tension lowering agents. Saponin, 0.1 gm. in intestinal loops, lowered the blood sugar 40%, without convulsions, and the same dose per rectum lowered it 67%, with convulsions. In mice, gastric doses of 0.01 gm. reduced the blood sugar 57%, with convulsions. Sodium lauryl sulfate, 0.1 gm. in intestinal loops, reduced the blood sugar 40%, and the same dose per rectum in rabbits, 53%, with marked convulsions. Bile salts, 0.01 to 0.1 gm. in intestinal loops, reduced the blood sugar 46%, with convulsions. The same doses per rectum in rabbits reduced the blood sugar 54%, with convulsions, but 0.01 gm. per rectum in rats increased the blood sugar 9%. Caprylic alcohol, 0.02 cc. in intestinal loops, reduced blood sugar 43%, with convulsions. Decholin, 0.1 gm. in loops reduced the sugar only an average of 22%, with occasional convulsions. Aerosol, which is claimed to be a powerful surface tension lowering agent, and a promotor of insulin absorption in dogs (8) was one of the most efficient promotors of insulin absorption from intestinal loops of rabbits, since 0.01 gm. reduced the blood sugar 58% and caused marked convulsions. Hexylresorcinol itself is not readily absorbed from the alimentary canal, but lowers surface tension in vitro and has been used successfully by Murlin and coworkers (7) for promoting gastrointestinal absorption of insulin in dogs. In intestinal loops of rabbits, 1 cc. hexylresorcinol in a solution of 0.1% strength was one of the least efficient agents tried, as the blood sugar was reduced only 11%, without convulsions.

Practically all agents, which lower surface tension, but especially saponin and hexylresorcinol, act as local irritants. However, the irritation from the volatile oils tried, was not important for promoting enteral absorption of insulin. By exclusion then, it would appear that the lowering of surface tension per se was the more important factor, or some cell surface effect on the intestinal mucosa in virtue of this physical property which facilitates the penetration of the insulin. This is not the only cell surface effect favorable to insulin absorption because astringents, alcohols, alcohol-amines, and anisotonic solutions also promoted absorption and the local physical changes from these agents are probably different.

Miscellaneous agents. Propermeability effects on different tissues have been claimed for caffeine, theophylline ethylenedimanine, and urea, but these agents in from 0.1 to 0.2 gm. doses in intestinal loops of rabbits also increased the blood sugar, contrary to a claim for gastric administration in dogs (7). The following agents, in intestinal loops, produced no demonstrable effects: brewer's yeast, peanut oil, lanolin (per rectum) piperazine, and pyridium. However, lecithin, 0.25 gm. in loops, lowered markedly the blood sugar, i.e., 64% with convulsions, contrary to a negative claim for this agent (11), fat-free colostrum (chiefly globulin) 0.5 gm. reduced it 31%, with moderate convulsions, methenamine, 0.3 gm. and sulfanilamide, 0.2 gm., reduced it about 10%, and kaolin, 2.0 gm., only 6%. From these results, it would appear that the mere presence of relatively insoluble or neutral substances, included fixed oils, in the intestine is not sufficient to affect cell surface activity favorable to insulin absorption. Some greater, or more severe, local cell surface activity appears to be necessary, as indicated in the previous section, in order to break down the natural barrier to enteral absorption of insulin. This leaves the matter a case of selective

permeability suspected previously by others. The other important factor, which can not be overlooked in oral administration, is the enzymic destruction of insulin, which must be arrested or inhibited in order that sufficient insulin might be exposed to the mucosa for absorption. In order to control this factor, certain antiseptics, but especially quinine, were found to be effective. With these general factors in mind, i.e., an antienzymic and a cell surface activity, there were selected the most promising and the safest agents from those used in animals for trials on normal men, including some agents tried by others.

Trials on normal persons. Seventy-one trials were made with 33 different agents and mixtures of these on 8 normal persons, the majority of the tests having been made on 2 persons. Most of the administrations were oral, some per rectum, and occasionally both methods with the same insulin mixture. The only criterion of insulin absorption was a hypoglycemia, which, in most cases, was variable and fleeting and lasted only about 1 to 3 hours.

The most consistent decreases in blood sugar were produced by quinine salts taken by mouth in doses of 0.5 gm. with insulin. The salts tried and the decreases in blood sugar were as follows: quinine sulfate, 15%; quinine hydrochloride, 7%; quinine tannate, 15%. Cinchonidine and quinine urea hydrochloride per rectum increased the blood sugar 50 and 100%, respectively, with considerable local irritation. The results with the quinine salts by mouth agreed qualitatively with those in animals, but the magnitude of the response in persons was much less. Most of the persons experienced the typical side-actions of quinine. Preliminary trials with insulin-quinine mixtures in a number of diabetic patients have sustained the results obtained in normal persons; these will be reported in a separate paper.

Next in order of hypoglycemic efficiency were the surface tension lowering agents, which also agreed with the results in animals. The decreases in blood sugar and the doses of the agents tried were as follows: 0.1 to 0.5 gm. saponin, 5% (oral) and 25% (rectal); 0.1 gm. saponin with 0.5 gm. saligenin as local anesthetic, 25% (rectal); 0.1 to 0.5 gm. bile salts, 3% (rectal) and the same with 0.05 cc. benzyl alcohol as local anesthetic, 12% (rectal); 0.5 gm. sodium lauryl sulfate, 5% (rectal) and 0.25 to 0.5 gm. aerosol, 1% (oral). Decholin, 5 gm. by mouth, increased the blood sugar 13% and bile salts with cholestrol produced no change. Most of these agents were found to be disagreeable, causing considerable local irritation, especially those given per rectum, and this was not relieved by the use of mild local anesthetics. Therefore, saponin and related agents were definitely unpromising for clinical use, contrary to the claims of Lasch and Schönbrunner, and despite their high efficiency in animals.

Moderate decreases in blood sugar were produced by the following comparatively mild antiseptics taken by mouth: 0.5 gm. thionol, 27%; 1 gm. methenamine, 8%; 0.5 gm. oxyquinoline sulfate, 12%. Sobisminol, 0.8 to 1.6 gm., caused a decrease of 6%, but the ingredients, other than bismuth, i.e., 10 cc. propylene glycol and 1.6 gm. triisopropanolamine, caused 4 and 8% increases, respectively. Mustard, 3 gm. with insulin, caused a decrease of 15% in blood sugar.

The following agents and mixtures taken orally, and some of which were antiseptics, caused increases in blood sugar from 1 to 38%: 0.1 gm. acriflavine, 0.25 gm. methylene blue, 0.25 gm. gentian violet, 0.75 gm. phenothiazine, 0.5 gm. benzoic acid, 0.2 gm. lecithin, 2 gm. lecithin and 2 cc. propylene glycol, 2 gm. lecithin and 2.1 gm. aerosol. The mixture of trypan red 0.1 gm. and malachite green 0.1 gm. with saponin 0.5 gm. recommended by Lasch and Schönbrunner (14) raised the blood sugar 25% and caused marked gastric irritation and discomfort. Accordingly, we were unable to confirm the positive claims of these authors. This dye-saponin mixture was quite as objectionable and unpromising for clinical use as was saponin or related agents

alone. Acetylsalicylic acid, 1 gm., and sodium nitroprusside, 0.3 gm., both taken orally, produced no changes in blood sugar, although general systemic effects occurred from both drugs. The negative results with acetylsalicylic agreed with those reported for animals.

SUMMARY

Mixtures of insulin and a variety of pharmacodynamic agents of known general properties and effects were tested for absorption in ligated intestinal loops and per rectum in rabbits, gastric administration in mice and rats, and oral and rectal administration in men. The criteria of insulin absorption were hypoglycemia and convulsions in animals and hypoglycemia in men.

The most effective and consistent promoters of insulin absorption in animals were surface tension lowering agents, alcohols, astringents, and a variety of protoplasmic poisons and antiseptics and the quinine salts. Simple hyperemia, foreign agents, and fats were ineffective. The important factors for enteral absorption of insulin appear to be enzymic inhibition and some local cell surface action, both being satisfied by the use of quinine sulfate.

Quinine with insulin by mouth resulted in definite, though fleeting, hypoglycemia in normal persons, thus confirming positive results in animals. Saponin alone, or in mixture with dyes, was too irritating and objectionable by mouth, or per rectum, for use clinically, contrary to claims by others.

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CLINICAL TRIALS WITH INSULIN-QUININE MIXTURES BY MOUTH IN DIABETICS[1]

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EXTENSIVE TRIALS of mixtures of insulin with a variety of agents in animals and men, showed that an insulin-quinine mixture given enterally, or orally, regularly produced hypoglycemia (1). In animals, this mixture produced convulsions, thus indicating a considerable absorption of insulin. When tried in human diabetics, the same quinine-insulin mixture frequently showed, though somewhat irregularly, beneficial effects. However, the results were encouraging enough to justify placing them on record in the hope that a search for agents in the light of the principles believed to be important for developing an oral absorbable insulin may be continued. This paper presents the results obtained in clinical diabetes.

The insulin mixture used for clinical trials contained 500 units of crystalline insulin[2] and 0.5 gm. quinine sulfate. The mixture was put into a gelatin capsule, and the capsule was coated with one of several substances used to increase its resistance to gastric juice. The substance most commonly used was a cellulose plastic material marketed as "enterab."[3] From 1 to 3 of these capsules were given daily, 45 minutes before meals, In certain trials, 10 cc. of alcohol, diluted, flavored and colored, was given with each quinine-insulin capsule for the purpose of further preventing destruction of insulin in the stomach. Sugar in urine and blood was estimated by Folin and Malmros's method (2). The 10 patients used were ambulatory cases, 2 being mild diabetics, and 8, with variable, though greater, severity of the disease.

Clinical Results

The essential results of the clinical trials are summarized individually as follows.

Patient 1. A woman 50 years of age, taking 12 U of regular insulin daily, was sugar-free in the urine and had a fasting blood sugar of 188 mg.%. After 1 capsule of insulin daily for 3 days her urine showed a trace of sugar, and the blood sugar was again 188 mg.%. She felt somewhat dizzy. This favorable effect was brief, but suggested a slight action of the insulin.

Patient 2. A man 74 years old, taking 12 U of regular insulin daily, was sugar-free and had fasting blood sugars of 122 and 135 mg.%. After taking one capsule of insulin daily for 4 days, his urine was sugar-free, and the daily blood sugars were between 134 and 146 mg.%. As in *patient 1*, this favorable result in a mild diabetic suggested that the insulin was effective. However, trials in more severe diabetics would be more critical, as in the following cases.

Patient 3. A man 66 years old, taking 32 U of protamine zinc insulin daily, was sugar-free with a blood sugar of 102 mg.%. After 7 days on 1 capsule of insulin daily, he was sugar-free with a blood sugar of 128 mg.%. On certain days the insulin was given by suppository, and on 2 occasions, relatives of quinine were substituted for quinine sulfate. Six months later, however, he was sugar-free while not taking any insulin, so the severity of this diabetes may have decreased spontaneously.

Patient 4. A woman 47 years old, who refused injections of insulin, showed consistent 2 and 3 + glycosuria with fasting blood sugars of 150 and 250 mg.%. On separate occasions she was tried twice on oral insulin. During the first period of 6 days she was given 2 capsules

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[2] The crystalline insulin was supplied by Eli Lilly & Co., Indianapolis, Ind.
[3] The enterab was supplied by the Abbott Laboratories, North Chicago, Illinois.

daily with a reduction in the glycosuria to sugar-free or 1 + tests, and of postprandial blood sugars of 150, 208 and 220 mg.%. A later period of 5 days with the same dose of insulin showed 1 to 3 + glycosuria with blood sugars of 200, 194 and 196 mg.%. Although the initial decrease in glycosuria was hopeful, this beneficial result was not duplicated in the second trial. However, during this time, she increased her diet greatly.

Patient 5. A woman 53 years old, taking 40 U of protamine zinc insulin daily, was sugar-free and had a postprandial blood sugar of 143 mg.%. With a change to 3 capsules of insulin daily, the blood sugar immediately rose to 250 mg.%, and there was a 3 + glycosuria. During the 5-day test period, however, her blood sugars were reduced to 178, 168, and finally to 105 mg.% and her urines to 1 + sugar. After a return to 40 U of protamine zinc insulin daily for 5 days, during which time her blood sugars were normal and urines sugar-free or 1 +, she was again put on 3 capsules of insulin daily for the next 10 days. Postprandial blood sugars were 164 and 172 mg.% and urines sugar-free or 1 +, which appeared to be fairly good controls for a moderately severe diabetic. For the next 124 days she was given no insulin in any form and her blood sugar gradually climbed up to 260 and 350 mg.%, with 3 + glycosuria, and she sustained a loss of 7 lb. in body weight. She was then given 3 capsules of insulin daily for the next 10 days, together with alcohol as above. Her blood sugars were 220, 220 and 135 mg.%, her urine was sugar-free or showed a trace of sugar, and she gained 2 lb. For the next 10 days she was given no insulin and her blood sugars rose to 280 and 340 mg.%, and she again developed 3 + glycosuria. During a final period on capsules, which contained the usual amount of quinine, but no insulin, plus the alcohol, her postprandial blood sugars were 225, 225 and 270 mg.%, but she was sugar-free or showed only 1 + glycosuria. This long and rather complicated trial showed the most favorable results obtained with oral insulin. This patient was never troubled with more than mild tinnitus, as a side-action from the quinine.

Patient 6. A woman 54 years old was sugar-free, with a blood sugar of 206 mg.% on 14 U of crystalline zinc insulin daily. She was given 2 and then 3 capsules of insulin daily, with considerable tinnitus and other distress. Her blood sugars rose, being 333, 328 and 363 mg.%, with 2 and 3 + glycosuria. After 10 days, the trial was terminated because of the toxic symptoms. Such an outcome is to be regarded as a failure from the standpoint of diabetic control.

Patient 7. A man 40 years old, taking 40 U of protamine zinc insulin daily, was poorly controlled, having a blood sugar of 303 mg.%, and varying glycosuria. He was given 3 capsules of insulin daily, but stopped them after 5 days, before the blood sugar could be estimated, because of extreme glycosuria, and mild cinchonism.

Patient 8. A woman 46 years old, taking 36 U of protamine zinc insulin daily, was sugar-free with a blood sugar of 175 mg.% after breakfast. She took 3 capsules of insulin daily together with alcohol for 12 days. On this regime, her blood sugars were 275, 225 and 280 mg.%, and glycosuria was 3 +. She was dizzy, weak, and menstruated 2 weeks before the expected time. Upon return to insulin injections, she quickly became sugar-free, and had a postprandial blood sugar of 170 mg.%. This patient is to be regarded as a complete failure as regards oral insulin therapy.

Patient 9. A man 51 years old, taking 40 U of protamine zinc insulin daily, was only moderately well controlled with 1 + glycosuria and a postprandial blood sugar of 150 mg.%. For 2 weeks he was given 3 capsules of insulin daily together with alcohol, the blood sugar being 225 mg.%, and the urine 3 + at the end of the first week, and 200 mg.% with no glycosuria at the end of the second week. During the next month he took no insulin and his urine sugar became 3 + with final blood sugars of 360 and 350 mg.%. During the next month he was again given capsules of insulin, but the blood sugars were 260, 280 and 250 mg.% with varying but severe glycosuria. Without insulin for the next 2 weeks his blood sugars were 325 and 300 mg.% with severe glycosuria, and during the next 2 weeks on quinine and alcohol without insulin his blood sugar remained high at 270 mg.%, although some urines were sugar-free. Treatment in this man can be regarded a partial success, for, although the diabetes was not entirely controlled by oral insulin, it was distinctly worse without insulin.

Patient 10. A woman 39 years old, taking 36 U of protamine zinc insulin daily, had 3 + glycosuria with postprandial blood sugars of 200 and 260 mg.%. During the next 3 weeks she took 3 capsules of insulin daily with alcohol. Although her blood sugars continued between 212 and 280 mg.%, glycosuria was only slight to 2 +. She had mild tinnitus. She had

no insulin for the next 3 weeks and showed more marked glycosuria, with blood sugars of 375 and 325 mg.%. This apparently indicated some effect from the oral insulin, but during the next 2 weeks, while taking the capsules again, she showed moderate glycosuria and blood sugars of 190 to 310 mg.%. One more week without insulin, and 2 weeks with quinine and alcohol, without insulin, gave blood sugars of 280, 375 and 320 mg.%, with varying severe glycosuria. In this case, the oral insulin produced slight beneficial effects, as the condition was worse without insulin.

DISCUSSION

Of the 10 diabetic patients given insulin-quinine mixtures by mouth in place of injected insulin, 3 with very mild diabetes were adequately controlled for brief periods. Because assessment of the degree of control in mild diabetics is difficult, particularly in short trial periods, later trials were made in moderately severe diabetics. In 7 such patients, the insulin mixture exhibited slight to considerable controlling effects in 4. On the whole, however, the effect was irregular and accompanied by the undesirable side actions of cinchonism in about one-half the patients. In the other 3 patients, the use of insulin mixtures by mouth was a failure. Since certain diabetics, particularly the elderly, have mild diabetes not requiring rigid insulin doses, and since they may also have trouble seeing the graduations on a syringe, it would seem that quinine-insulin mixtures by mouth might have occasional clinical use.

The comparatively low efficiency of this oral insulin mixture, in which 1500 U daily may not control a moderately severe diabetic, greatly limits an immediate use of such a mixture, in the treatment of the ordinary diabetic. On the other hand, it is fairly encouraging to have discovered an agent which depresses ferment destruction of insulin and the selective absorbing intestinal cells and thus promotes the absorption of insulin, and also permits therapeutic trials with comparative safety. In the incomplete trials made by us, it appeared that quinine alone may possibly have some slight action in decreasing glycosuria, but has no demonstrable effect on blood sugar.

The chief objection to the use of quinine, of course, is the occurrence of cinchonism. Although this symptom complex is unpleasant and not particularly harmful and the individual variation in reaction is considerable, this undesirable feature must be eliminated in the use of insulin by mouth. It would seem to be in order to search for related substitutes for quinine until a suitable agent is found. The 2 chief properties to be sought in such an agent are antifermentative and propermeability actions in the intestine. Attempts should also be made to reduce the dose of insulin for sake of economy.

CONCLUSIONS

A majority, or 7, of 10 patients, with mild and severe forms of diabetes, reacted beneficially, when given variable doses of an insulin-quinine mixture by mouth; in some cases the mixture was given with some alcohol.

About one-half the patients showed the well known side-actions of cinchonism in variable degree.

The results obtained are to be regarded as encouraging concerning future possibilities of discovering more efficient and less objectionable agents than quinine for promoting gastrointestinal absorption of insulin. Meanwhile, the insulin-quinine mixtures used by us are not recommended for general clinical use.

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QUANTITATIVE PRECIPITATION OF THE URINARY GONADOTROPIN OF NORMAL MEN AND WOMEN[1]

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METHODS for the quantitative concentration of urinary gonadotropic substances, being of primary importance in the determination of the rate of excretion of such substances, have attracted considerable attention in the past few years. Particularly interest has been attached to the application of such methods to the urines of normal individuals which, because they contain very small amounts of active material, must be concentrated into proportionally small volumes of nontoxic extract in order to be suitable for all animal assay.

Several years ago, on the basis of experiments with the urines of post-menopausal and ovariectomized women, we (1) suggested that tannic acid is an effective precipitant for urinary gonadotropin. That the precipitation is quantitative was determined by comparing the yields obtained by this method with those by assay of the raw urine or of alcohol precipitates thereof. The alcohol precipitation is generally assumed to be and probably is quantitative but, as is well known, extracts prepared in this way cannot be administered in amounts equivalent to large quantities of urine, as is necessary in the case of normal urines, because of the great toxicity. For this reason a comparison of yields from normal urine by tannic acid and by alcohol precipitation was not possible. However, on the basis of yields obtained by tannic acid precipitation as compared to those obtained by certain other methods, it was suggested that tannic acid is a suitable and effective precipitant for the gonadotropin or normal urines. This has been confirmed by others (2–7). Recently, however, Heller and Heller (8), on the basis of results obtained with menopausal urine, have questioned whether tannic acid precipitation is quantitative. It is indeed evident from the data given by these authors that they recovered less activity by tannic acid than by alcohol precipitation.

Because of the complete disagreement of these results and those obtained in this and other laboratories, we undertook the present work, a comparison of yields obtained from the urine of normal men by tannic acid and by alcohol precipitation. The results, presented in this communication, amply confirm our earlier statement concerning the efficacy of the tannic acid method and also demonstrate some of the pitfalls to be encountered in excessive manipulation of extracts containing small amounts of gonadotropin. Full details of the tannic acid method are given in the hope that others may use it with equal success.

EXPERIMENTAL

Tannic acid precipitation. Because unpreserved urine may rapidly develop toxic properties, all urines are collected directly in bottles containing small amounts of chloroform. After thorough chilling (5°C.) the cold urine is brought to pH 4.5 to 5.0 (methyl red or brom cresol green) by addition of glacial acetic acid. All indicators are used externally, adding a very small drop of indicator solution to one or two drops of the test solution on a spot plate. The freshly prepared aqueous tannic acid solution is immediately added, 50 cc. of 20% solution usually being sufficient for a 24-hour urine

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specimen.[2] An immediate precipitate forms. The mixture is thoroughly stirred and is allowed to stand in the refrigerator for 30 to 90 minutes. The supernatent solution is syphoned off, portions of it being used to quantitatively transfer the precipitate into 250 cc. centrifuge bottles. The supernatent is also used to facilitate the collection of the precipitate into one centrifuge bottle after the centrifugation, which need only be of very short duration. In fact, if centrifuged too long the precipitate has a tendency to pack too hard, rendering subsequent treatment more difficult. In this way the entire volume of urine is centrifuged. The precipitate, now in one centrifuge bottle, is immediately suspended in 200 to 250 cc. of 95% ethanol and all lumps are completely broken up. If this ethanol extraction is delayed the precipitate tends to become gummy and difficult to handle. The mixture is centrifuged and the insoluble residue is twice thoroughly extracted with 80% ethanol, using 250 and 100 cc. volumes respectively. It is then washed with a small amount of acetone followed by a washing with ether and finally is freed of residual ether by reduced pressure. The bottle containing the dry precipitate is stoppered and stored until assay.

The dry powder is prepared for assay by extraction with dilute alkali at pH 8.5 to 9.0 (phenolphthalein). The final volume of the extract is calculated (dependent on the volume of urine and the concentration of gonadotropin expected) and slightly less than one-third of this volume of water is added to the pulverized precipitate. Dilute alkali[3] (0.5 to 1.0 N NaOH or NH_4OH) is now added dropwise until pH 8.5 to 9.0 is atained. The mixture is thoroughly stirred so all particles are completely broken up and the insoluble residue removed by centrifugation. This residue is similarly re-extracted two more times, any material remaining insoluble after the third extraction being discarded. The three alkaline extracts are combined and adjusted to pH 7.5 (phenol red) by dropwise addition of dilute acetic acid. The extract is now diluted to the calculated volume and suspended material, if any, removed by centrifugation. This extract is used for injection and is stored in the refrigerator at all times. We prefer to keep the activity in extract form for as short an interval as possible. Therefore we retain it as the dry tannate and do not make the final solution until the day before injections are to begin.

Alcohol precipitation. The alcohol precipitation method, as employed by us, was the familiar Zondek method, i.e., precipitation by addition of 5 volumes of 95% ethanol to the urine at pH 4.5 to 5.0 (acetic acid). Because the extracts prepared from the alcohol precipitates are too toxic for use in the large doses required when normal urines are being assayed, we employed the dialysis procedure recommended by Heller and Heller (8). The extracts of the crude alcohol-precipitated, ether-washed and vacuum-dried material, prepared by leaching the dry precipitate 3 or 4 times with dilute alkali at pH 8.0 to 8.5, were transferred to cellophane bags (Visking) and dialyzed against running tap water for periods varying from 4 to 48 hours. The temperature was always below 20°C. The volume of the solution was always considerably increased during dialysis, necessitating reconcentration to an appropriate volume. The reconcentration was effected by air evaporation (Heller and Heller) to the desired volume or by acetone precipitation. The latter procedure was carried out by adding 10 volumes of acetone and several drops of ammonium acetate solution to the dialyzed solution and chilling over night. The precipitate was then collected at the centrifuge, washed with acetone and ether and freed of ether by reduced pressure. On the day preceding assay the precipitate was dissolved in water and the pH of the solution adjusted to 7.5.

[2] We are indebted to Dr. H. W. Sulkowitch for the suggestion that the amount of tannic acid previously recommended by us (20 cc. of a 10% solution per liter of urine) is not sufficient to completely precipitate some urine specimens.

[3] For reasons which space does not permit elaborating, we prefer to use NaOH or NH_4OH rather than a buffer mixture as the extraction medium.

TABLE 1. YIELD OF GONADOTROPIN FROM URINE OF NORMAL MEN

Subject and age	24-hour gonadotropin excretion by		Recovery by alcohol ppt'n	Treatment of extract of alcohol precipitate		
	Tannic acid	Alcohol ppt'n		Dialysis		Reconcentration
	M.U.U.	M.U.U.	%[1]	Hr.	°C.	
E.C.W. 37	20	15	75	40	18	Acetone ppt'n
L.L. 31	25	15	60	48	18	Acetone ppt'n
	24	15	62	40	18	Acetone ppt'n
	25	15	60	40	20	Acetone ppt'n
	20–25	15	60–75	24	20	Acetone ppt'n
	25	10–15	40–60	24	19	Acetone ppt'n
G.K.S. 30	20	15	75	24	19	Acetone ppt'n
	20–24	10	40–50	24	23	Acetone ppt'n
	15–20	15–20	100	4	12	None
J.H.L. 28	20	<7.5	<38	24	20	Acetone ppt'n
	15–20	7.5	38–50	24	19	Acetone ppt'n
	<10	<7.5	?	24	23	Acetone ppt'n
	10–15	<7.5	<75	6	16	Evap. 40%
R.C.C. 28	10	10	100	48	17	Acetone ppt'n
	12	<7.5	<60	48	23	Acetone ppt'n
	10–15	10–15	100	5	10	None
E.J.M. 27	15	15	100	5	12	Evap. 26%
	24	24	100	5	10	None
J.W.J. 25	10–15	<7.5	<75	48	23	Acetone ppt'n
	<10	10	>100	24	23	Acetone ppt'n
	<7.5	7.5	>100	5	15	Evap. 50%
Pooled	24–30	15–20	50–80	24	19	Acetone ppt'n
	20–25	15	60–75	4–5	15–20	None

[1] Yield by tannic acid assumed to be 100%.

Assay method. Assays of gonadotropic potency were made by the mouse uterine technique as previously described (9). It must be emphasized that our assay method depends on determining the smallest dose which produces the required 150% increase in uterine weight and not on interpolation from a predetermined dose-response curve. Thus each extract is assayed at a number of dose levels. For example, the extract of the urine specimen is made up to a final volume so that 18 cc. are equivalent to 24-hour output. The extract is then assayed in mice at total dose levels of 0.60, 0.90, 1.20, 1.80 and 2.40 cc. corresponding to 30, 20, 15, 10 and 7.5 M.U.U.[4] per 24 hours. If the 1.20 cc. dose produces the required response (150% increase in uterine weight) while the 0.90 cc. does not produce the response, we calculate that the urine contained at least 15 but less than 20 M.U.U. per 24-hour specimen. As many mice as possible are used at each dose level, the amount of urine used being the limiting factor.

RESULTS

Recovery from normal male urine. A comparison of the yields obtained by tannic acid precipitation and by alcohol precipitation-dialysis was made on 21 samples of urine collected from 7 healthy men 25 to 37 years old and on two specimens of pooled urine of medical students. The individual specimens were collected over 72-hour periods. After chilling over night, the urine was decanted from the chloroform pre-

[4] M.U.U. = mouse uterus units.

servative, thoroughly mixed and divided into two equal aliquots, each representing half of the 72-hour urine output. One aliquot was immediately precipitated with tannic acid as described above. At the same time the second aliquot was precipitated by alcohol. Both preparations were worked up rapidly and stored as dry powders until animals were available for assay. The assays on companion preparations were made at the same time, in order to render conditions as identical as possible.

The results (table 1) show that the tannic acid preparations actually contained more gonadotropic activity than did the corresponding alcohol preparations. In 20 of the 23 comparisons the tannate contained as much as or more activity than did the alcohol precipitate. Two samples showed a greater amount of activity in the alcohol precipitate and one sample was negative by both methods.

The actual quantities excreted varied among individuals but was fairly constant for each individual, although more data are required on this point. It is to be recalled that these determinations were made on aliquots of 72-hour urine specimens, which would tend to minimize day to day variations if such occur. However, it is interesting to note that 20 of the 23 values obtained by tannic acid precipitation fall between the limits of 10 and 25 M.U.U. per 24 hours.

Losses by the alcohol method. In the light of previous comparisons of the efficacy of the two methods in precipitating the gonadotropic activity of the urine of castrate and postmenopausal women, the greater activity of the tannates as compared to the alcohol precipitates in the present experiments was surprising. However, the additional purification procedures required for the alcohol precipitates of normal urine and not for those of urine of greater activity suggested that losses might be incurred in these procedures. An investigation was therefore made of possible loss of activity from the alcohol precipitates by the procedures used.

The results of the investigation are shown in table 2. An aliquot of the pooled urine of young men (*L614*), precipitated by tannic acid, contained 20 to 25 M.U.U. per liter of urine as indicated by 6 separate assays of the dry tannate. Another aliquot of the same urine was precipitated simultaneously by alcohol and dried. In 5 experiments, aliquots of this alcohol precipitate, extracted and dialyzed but not reconcentrated, contained 15 M.U.U. per liter of urine or only 60 to 75% of the activity found in the tannic acid precipitate.

TABLE 2. YIELD OF GONADOTROPIN FROM POOLED URINE OF NORMAL MEN

Preparation	No. exps.		Response at indicated dose levels					Yield, %
			30 M.U.U.[3]	25 M.U.U.	20 M.U.U.	15 M.U.U.	10 M.U.U.	
Tannate	6	Uterine wt.[1]	10.5	13.6	15.2	26.4	34.7	100
		% positive[2]	21	33	55	85	100	
Extract of alcohol precipitate								
Dialyzed	5	Uterine wt.		8.3	10.2	16.0	29.9	60 to 75
		% positive		11	19	62	96	
Dialyzed and evaporated	2	Uterine wt.			9.9	13.3	21.2	60
		% positive			0	44	90	
Dialyzed & acetone ppt'd.	2	Uterine wt.			6.6	9.0	21.9	50
		% positive			0	10	90	

[1] Average uterine weight in mg.
[2] Animals whose uteri weigh 13 mg. or more.
[3] M.U.U. = mouse uterus units per liter of urine.

TABLE 3. GONADOTROPIN OF URINE OF OVARIECTOMIZED WOMEN. RECOVERY AFTER VARIOUS PROCEDURES

Procedure	No. exps.		Response at indicated dose levels						Yield %
			15 mg.	20 mg.	22.5 mg.	25 mg.	30 mg.	37.5 mg.	
Extract of alcohol ppt.	8	Uterine wt.[1]	9.1	13.6	15.8	25.8	29.9	34.0	100
		% positive[2]	7	52	55	90	100	91	
Dialysis	6	Uterine wt.		7.6	9.6	12.6	13.7	24.7	67
		% positive		0	15	20	46	87	
Dialysis and evaporation	4	Uterine wt.			5.7	8.7	10.3	15.7	55 to 60
		% positive			0	20	26	61	
Dialysis & acetone ppt'ion.	3	Uterine wt.				8.2	13.1	17.4	67
						0	50	75	

[1] Average uterine weight in mg.
[2] Animals whose uteri weigh 13 mg. or more.

In two experiments, reconcentration by evaporation of the dialyzed extracts of the alcohol precipitate resulted in a further slight decrease in activity as indicated by lower uterine weights and smaller percentage of positive responses at the same dose levels. In these experiments the evaporation, effected by blowing a gentle stream of air over the surface of the solution (PH 7.5; temp. 35–40°C.) amounted to about 40% of the original volume.

Another source of error encountered was that of incomplete extraction of the gonadotropin from the bulky alcohol precipitate. As mentioned above, the precipitate was extracted at least three and usually four times with fresh portions of dilute alkali. The major portion of the solid material remains insoluble and evidently retains an appreciable portion of the gonadotropin as indicated by the following findings. In several experiments the insoluble residue remaining after four extractions was further extracted three times with fresh portions of alkali. These last three extracts were combined, dialyzed, reconcentrated and assayed. In each case an amount of activity equal to at least 10% of the total was found in the extracts. That this is not entirely unexpected becomes evident from a careful consideration of the procedure. The dried alcohol precipitate from a 24-hour urine specimen weighs 4 to 6 gm. Most of this precipitate consists of inorganic salts. When the large bulk of solids is extracted with the relatively small quantity of water which may be used, it is readily seen that the extract contains quite a high concentration of salt, thereby decreasing the solubility of the gonadotropin, which is relatively insoluble in concentrated salt solutions.

A comparison of activity before and after dialysis of extracts of alcohol precipitates of normal urines is not possible because the undialyzed extracts are not tolerated by the assay animals in the large doses required to produce a positive response. Therefore, to test for loss during dialysis we utilized an alcohol precipitate of higher activity (*L610*) prepared from the pooled urines of castrate women several years prior to the present experiments and stored as the dry precipitate until the present time. For this reason, no corresponding assay of the tannate of the same urine is available.

In 8 separate assays (table 3) it was found that 20 to 22.5 mg. of this powder were equal to 1 M.U.U. if no dialysis was performed. In 6 experiments the extracts of the powder were dialyzed but not reconcentrated. The activity was 30 mg. per M.U.U., a loss of 33%.

Similarly, in 4 experiments, the activity of the dialyzed extracts after removal of 20 to 60% of the solvent by air evaporation was about 37.5 mg. per M.U.U., a loss of 20% on the basis of the dialyzed non-evaporated extracts or of about 40 to 45% on the basis of the non-dialyzed extracts. In 3 experiments the reconcentration of the dialyzed extracts by acetone precipitation resulted in a loss slightly less than that incurred by air evaporation.

The data obtained from these experiments thus explain why the alcohol precipitation method yields satisfactory results when applied to urines of higher activity (castrate, post-menopausal) requiring no purification procedures but low values when applied to normal urines which require additional purification. It also becomes apparent that for determination of normal gonadotropin excretion tannic acid precipitation is superior to the alcohol method.

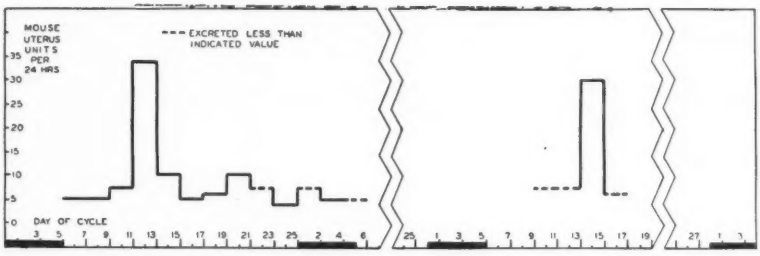

Fig. 1. Gonadotropin excretion during normal menstrual cycle. Subject E.B.H.

Excretion by normal women. It is now known that normal women excrete as little as and frequently less gonadotropin than do normal men. To demonstrate the efficacy of the tannic acid method for concentration of the urinary gonadotropin of normal cyclic women, data obtained from two subjects are presented.

Consecutive 48-hour specimens of urine were collected over at least a complete menstrual cycle in each case. Forty-eight hour rather than 24-hour samples were used so as to provide sufficient material for complete and dependable assays. The data are presented in figures 1 and 2.

Subject 1 (*E.B.H.*) was a normal woman, nullipara, age 21 years at the time of the experiments. Her menstrual cycle was regular, varying around a mean of 28 days. The cycle during which complete determinations were made was 25 days long. As may be seen from figure 1, this subject excreted less than 7.5 M.U.U. per 24 hours, except at two points in the cycle. A maximum of 35 U appeared on days 12 and 13 (after the beginning of menses). This increase in excretion was very rapid and was of short duration, 48 hours or less, since the sample preceding and the one following assayed near the base level.

The second peak was very slight, a level of 10 U being found on days 20 and 21, 8 days after the first sharp peak. Whether this rise has any significance is doubtful. It may, however, correspond to the secondary peaks observed by D'Amour et al. (4).

During a subsequent cycle, determinations of the daily excretion of this subject were made over a period of 8 days when the peak in excretion was expected. As indicated in figure 1, the increased excretion again appeared, the days of the increase being almost identical with those found in the first cycle.

Subject 2 (*E.H.*) was a normal nulliparous married woman 28 years old. Her menstrual cycle was regular, varying between 30 and 34 days. As shown in figure 2, for the first 22 days of the cycle this subject excreted less than 7.5 U per 24 hours except

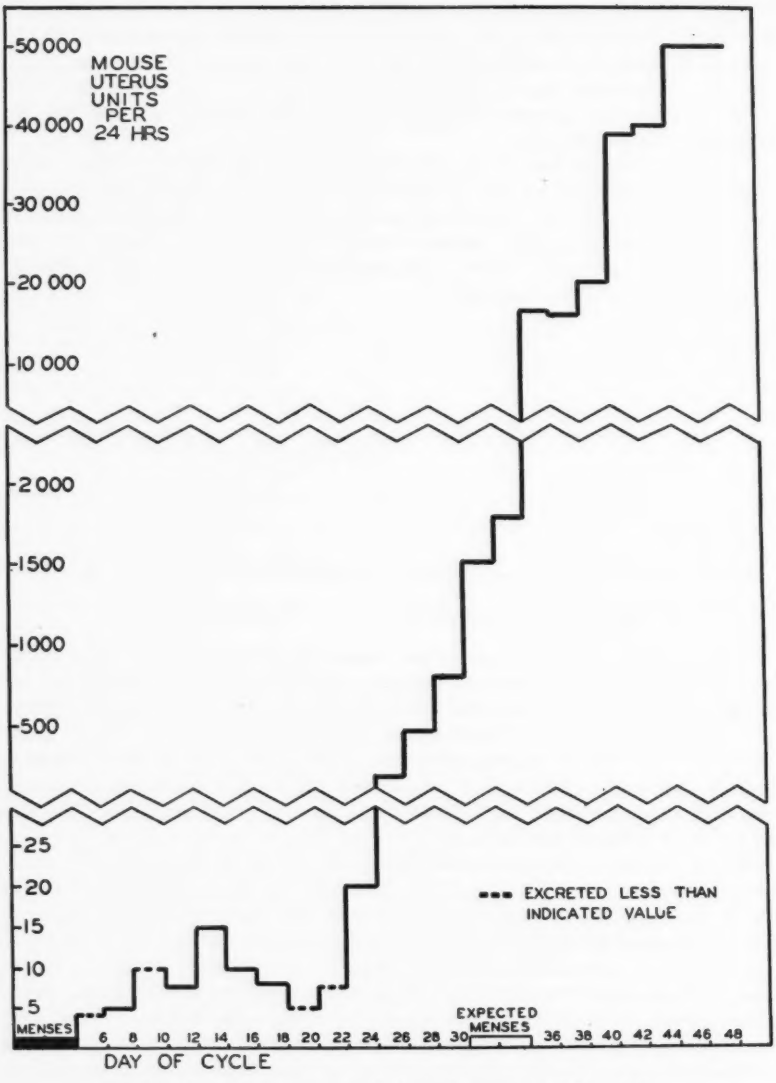

Fig. 2. Gonadotropin excretion during early pregnancy. Subject *E.H.*

for one maximum on days 13 and 14 when the excretion rose to 15 M.U.U. It is possible that this peak may have been higher had 24-hour rather than 48-hour specimens been assayed. It is interesting that the days of the peak in this case closely correspond with those of case 1.

On the 23rd day after the beginning of the last menstrual period the urinary gonadotropin of this subject began to rise and showed a very rapid increase as long as determinations were continued. Blood points and corpora were noticed in the

ovaries of the assay mice and it became obvious that the subject was pregnant. The expected menses should have appeared on day 30 to 32. The subject delivered a normal child 295 days after the beginning of the last menses.

Toxicity. The objection has been made (8) that tannic acid preparations are toxic. It is true that large doses often prove to be toxic to the assay animals. However, it must be remembered that large doses of urine extract, no matter how prepared, are toxic. Of all the concentration methods which have come to our attention, tannic acid precipitation yields the least toxic preparations.

While performing the experiments reported in this communication, the following experience was gained (table 4). Of 90 mice which were injected at the 5 U level (1/5 of

TABLE 4. TANNIC ACID PRECIPITATE OF URINE OF NORMAL ADULTS. INCIDENCE OF TOXIC SYMPTOMS IN MICE

Subject	Fraction of 24-hour urine specimen injected									
	1/5 No. of mice		1/7.5 No. of mice		1/10 No. of mice		1/15 No. of mice		1/20 No. of mice	
	total	*toxic*	*total*	*toxic*	*total*	*toxic*	*total*	*toxic*	*total*	*toxic*
Female 1	38	15	15	6	38	1	36	0	33	0
Female 2	52	21	37	6	52	4	50	1	46	0
Males (7)			38	15	67	2	112	5	109	1
Totals	90	36	90	27	157	7	198	6	188	1
Toxic symptoms	40%		30%		4.4%		3%		0.5%	

the 24-hour output per mouse divided into 3 equal doses over a 3-day period) 36 (40%) showed definite toxic symptoms or death. The remaining 54 mice (60%) showed no toxic symptoms. Of 90 mice injected at the 7.5 U level 63 (70%) showed no toxic symptoms while 27 mice (30%) either died or showed toxic symptoms during the injection period. It should be emphasized that these include mice injected with preparations of urine excreted during the menstrual flow. Preparations made from such urines are extremely toxic even at lower doses. Of 157 mice injected with one-tenth of the 24-hour output (10 M.U.U./24 hours) only 7 (4.5%) died or showed signs of toxicity. At lower doses (e.g., 15, 20, 25, 30 M.U.U. per 24 hours) practically none of the assay mice showed toxic symptoms. The preparations used were derived from urines of the 8 individuals mentioned above.

Although the toxicity of large doses of tannic acid preparations, as indicated by the above figures, is annoying, it must be remembered that with other types of unpurified preparations (e.g., alcohol precipitates) the toxicity at such dose levels would be almost 100%. In experience gained several years ago, it was noted that most assay mice died if injected with unpurified extracts of alcohol precipitate equivalent to more than 50 cc. of urine. Thus one could test for no less than 20 M.U.U. per liter of urine. This experience is not unique and similar results have been obtained in many other laboratories.

A word should be added concerning the remarks of Heller and Heller (8) regarding the relative activity of suspensions and solutions of the urine tannate. It is now common knowledge that in many instances the pharmacological action of a drug may be enhanced or changed by decreasing the rate of absorption from the site of injections. The prolonged action of protamine-insulin combinations is well known. Similarly the gonadotropic action of pituitary preparations may be enhanced or changed by decreasing the absorption rate (10, 11, 12). It seems not unlikely that the same explanation applies to the greater activity of the undissolved tannate as reported by Heller and Heller (8).

It should be emphasized that we dissolve the tannate rather than to 'elute' it. We are of the opinion that tannic acid forms a definite insoluble chemical combination with the gonadotropin and the latter may be rendered soluble only by bringing the pH above the isoelectric point. We have not been able to extract any activity from tannates by solvents other than those having an alkaline reaction. Neither have we been able to find any activity in the small amount of insoluble residue which remains after several extractions with alkali.

DISCUSSION

For the quantitative determination of the normal excretion of gonadotropin, a satisfactory concentration method must meet three prerequisites: *a*), it must be quantitative; *b*), the extracts produced must be sufficiently non-toxic so that large quantities of urine may be injected into the assay animals; and *c*), it must be simple and rapid so that large numbers of urine samples may be handled if necessary. That the tannic acid precipitation method meets these prerequisites to a greater extent than any other method proposed seems to be established by the experiments presented here and previously published.

The experiments performed in this laboratory have definitely convinced us that if tannic acid precipitation is properly done, the resulting extracts contain at least as much of the original urinary gonadotropic material as may be obtained by any other method. This is true for the urine of postmenopausal or castrate women as well as for the urine of normal men and, as far as is known now, this is also true for the urine of normal women. Of all other concentration methods which have come to our attention, the original unmodified alcohol method of Zondek is probably the easiest and most nearly quantitative. No claim is or has been made by the writer that the tannic acid method surpasses the alcohol precipitation method in either of these two qualities, and it has always been frankly admitted that if possibly applicable the alcohol method is the more advisable. However, as stated above, and as experienced by anyone who has attempted to apply the alcohol method to normal urines, toxicity makes it impossible to inject sufficiently large doses to test for the small amounts of activity present. Most workers have encountered difficulty from this source in determining the gonadotropin excretion of normal men. Since the urine of normal women contains even less gonadotropin (except for 1 or 2 days during the cycle) than does that of men, it becomes obvious that with such urines (of women) it is practically impossible to inject enough of the extract to get a positive response. This leaves no doubt that the alcohol method, without modification, cannot be used for the assay of gonadotropic activity of urines of low activity such as those of normal men and women. This statement is supported by the work of Katzman (2) who found the unmodified alcohol extracts of normal male urine too toxic for assay and of Heller and Heller (8) who found that when studies of gonadotropic excretion of normal men and women were attempted, the unmodified alcohol extracts proved too toxic for use. Heller and Heller state that toxicity seemed to be "limited to urines of young adults and was not encountered in working with menopausal urines." This, of course, is to be expected, since extracts of the latter equivalent to only a few cc. of urine need be injected, whereas with normal urines (young adults) extract equivalent to large amounts of urine must be injected.

It is therefore apparent that alcohol precipitation, if to be used for determination of normal gonadotropic excretion, must be modified in some manner so as to reduce the toxicity of the extracts. A number of methods of accomplishing this have been suggested by various investigators, the latest and probably the most effective being the dialysis procedure of Heller and Heller. It is quite true that dialysis does substantially decrease the toxicity of the extracts. However, because of the increase in

volume during dialysis it becomes necessary to reconcentrate the dialyzed solution. This may be accomplished by evaporation by a stream of air as suggested by Heller and Heller, or by precipitation with a large volume of acetone (or alcohol) as was done in some of the experiments reported in this communication. Thus several additional manipulations are introduced, making the method considerably more laborious and time consuming. More important, however, is the great possibility of loss of activity during the added manipulations, either by chemical destruction or by mechanical loss. This is shown by the results presented in this communication for in 15 of 23 comparative determinations the yield by alcohol precipitation-dialysis was definitely and significantly lower than that obtained by tannic acid precipitation. It has furthermore been demonstrated that definite and appreciable losses of activity do occur during the manipulations incident to dialysis and reconcentration of the extract. Also, it is almost impossible to extract all of the active material from the voluminous alcohol precipitate unless proportionally large quantities of extraction medium are used. This is probably of even greater importance when dilute alcohol or acetone solutions are used as extraction mediums.

The situation at the present time therefore amounts to a choice between using alcohol precipitation followed by a purification to reduce toxicity, with the probability of a very appreciable loss of activity, or of using some other method (such as tannic acid precipitation) which does not entail purification procedures after the initial precipitation. Extracts prepared from menopausal or castrate urines by tannic acid precipitation have been shown to contain all the activity of the original urine which can be demonstrated by any method. Tannates from normal male urine have been shown in this communication to contain more activity than do the alcohol extracts detoxified by dialysis. The tannic acid method is certainly less laborious and time consuming than is the alcohol method plus the necessary purification. Finally, the toxicity of the tannic acid extracts (if well preserved urines are used) is not sufficiently great to interfere with the assay of urines containing as little as 5 or 7.5 mouse uterus units per 24-hour specimen. We therefore maintain our previously expressed opinion that tannic acid precipitation is a very effective procedure for the quantitative determination of the gonadotropic excretion of normal men and women.

SUMMARY

Tannic acid precipitation recovers more gonadotropic activity from the urine of normal men than does alcohol precipitation followed by the necessary detoxication of the precipitate.

The low yields obtained from normal urines by alcohol precipitation are due to incomplete extraction from the large bulk of precipitate as well as to losses during dialysis and reconcentration of the extract.

Tannic acid precipitates, without purification, are sufficiently non-toxic to permit determination of the gonadotropin content of the urines of normal men and women.

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CYTOLOGIC AND HISTOLOGIC OBSERVATIONS ON THE EPITHELIAL, CONNECTIVE AND VASCULAR TISSUES OF THE ENDOMETRIUM OF MACAQUES UNDER VARIOUS EXPERIMENTAL CONDITIONS[1]

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WITH INCREASING INTEREST in the use of the endometrial biopsy specimen as an aid in the diagnosis of ovarian and menstrual disorders, the necessity for a more exact knowledge of the action of the ovarian hormones upon the various tissues comprising the endometrium becomes apparent. The *Macacus rhesus* monkey has been employed for observations and experimental work on the reproductive cycle of primates because of the similarity of the menstrual cycle and of the uterine tissues in the macaque and in the human being.

A review of the literature to obtain information regarding the structure of endometrium in castrated macaques when treated with preparations of the ovarian hormones, revealed that few papers were primarily concerned with this phase of study of the problems related to reproduction. Some investigators (1–5) briefly described the essential characteristics of the endometrium of castrated animals, as well as those of the tissue from the castrated macaques treated with estrogen. Others (6–10) have added some information regarding glycogen and subnuclear vacuoles in tissues from castrated animals treated with estrogen. Hisaw, Meyer and Fevold (11) first used preparations of the corpus luteum combined with estrogenic substances in castrated macaques. Hisaw (5), Lendrum and Hisaw (7), Rossman (12), Hisaw and Greep (13), and Rossman (14) have contributed the information available regarding the action of the combined ovarian hormone preparations upon the endometrium of castrated monkeys. Hisaw (5) and Lendrum and Hisaw (7) described the action of corpus luteum hormone upon the endometrium of castrated animals which had not been primed with estrogenic substances.

The anatomy and physiology of the vascular bed of the endometrium of *Macacus rhesus* has been described by Daron (15) and Markee (16), respectively. Van Wagenen and Zuckerman (4) and Hisaw (5) called attention to the dense stroma in the endometrium of castrated and untreated macaques. The Golgi apparatus has bene observed under the known influence of the follicle hormone, and of the follicle and corpus luteum hormones combined (5, 7, 14). The epithelial proliferations resulting from traumatization of endometrium in castrated animals under the influence of the follicle and corpus luteum factors were noted (17) and described at length (14). The latter paper reported cilia on the gland cells in the endometrium of experimental animals, while mucin and fat were found by Lendrum and Hisaw (7) in tissues from castrated animals treated with ovarian hormone preparations. The matter of the occurrence of subnuclear vacuoles and of glycogen in the endometrium of experimental animals has received some attention. The vacuoles as structures in experimental tissues were first reported (4) as occurring in castrated animals treated with estrogenic substances only. Hisaw (5) and Lendrum and Hisaw (7) believed the presence of these structures was characteristic

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of the action of the corpus luteum factor upon endometrial tissue, while Overholser and Nelson (8) showed that the structures occurred in the endometrium of castrated animals treated with estrogenic factors exclusively. Zuckerman and Morse (6) described vacuoles in the epithelium of endometrium from castrated animals treated for prolonged periods of time with high dosages of estrogenic material, and believed they represented an indication of incipient cell degeneration in the tissues studied. Glycogen was described in endometrial tissues from castrated animals receiving the ovarian hormones (2, 7, 10, 13, 14).

The chief means of differentiating tissues secured from castrated untreated animals and those from castrated animals treated with known amounts of the follicle and corpus luteum factors is based primarily on the depth of the endometrium from myometrium to uterine lumen, variations in gland form, the position of the nuclei in the cells of the gland, and, to some extent, on the density of the stroma.

The present report is concerned with a systematic histologic and cytologic description of the epithelial, connective and vascular tissues comprising the endometrium of castrated macaques under known conditions of hormonal stimulation. In order that the study should be of practical value, the tissues have been prepared in a mode identical with that employed in the clinical laboratory of the Department of Obstetrics and Gynecology of Vanderbilt University Hospital. These findings do not answer many of the questions on endometrial structure, but they may prove of value in interpreting endometrial structure in terms of ovarian activity in normal and pathologic specimens of human endometrium.

MATERIAL AND METHODS

The material upon which this report is based was originally prepared for microscopic study in order to observe the reaction of endometrial nuclei to various hormonal stimuli. A report of these findings, including the details of the experimental procedure appeared recently (18).

Briefly, it may be said that the tissues were secured following castration (table 1), treatment of castrated monkeys with estrogen alone[2] (table 2), progesterone alone,[2] (table 3), and estrogen and progesterone in combination following a period of priming with estrogen (table 4).

All biopsies were secured within 24 hours following the last injection of hormone substances. The tissues were fixed immediately in Bouin's fluid, and stained with hematoxylin and eosin in the same routine laboratory procedure as that employed for human tissue in the Department of Obstetrics and Gynecology.

A monocular microscope with an apochromatic oil immersion lens of 100× magnification and compensating oculars of 15× and 25× magnifications were employed in this study.[3]

FINDINGS

A description of endometrial structure following various experimental procedures may become tedious because of the variety of tissues comprising the endometrium itself, and their variety of response to hormonal stimuli in different amounts and combinations for different periods of time. In the descriptions which follow, the tissue characteristics of glands and stroma for each group of experiments will be given first, followed by observations on the cytology of the gland and stromal cells, and on the vascular bed.

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[2] The estrogen, as progynon-B, and progesterone, as proluton, were generously supplied by Dr. Erwin Schwenk of the Schering Corporation.

[3] The source of light for the microscope was a gooseneck table lamp with an ordinary plain glass bulb, but a daylight blue bulb gave a better light for this purpose. The substage diaphragm was constantly in use to vary the amount of illumination of the cytoplasmic structures.

Endometrium from Castrated and Untreated Macaques

The specimens of endometrium from this group of animals were obtained at intervals of 3, 4, 5 and 6 weeks following removal of the ovaries. They have been divided into 2 groups for purposes of description, since the cytologic detail varied. The differences appeared to be associated with the presence or absence of lutein tissue in the ovaries at the time of castration (table 1).

General tissue characteristics. The general histologic characteristics of the specimens of endometrium from castrated and untreated macaques were similar, regardless of the type of ovary at the time of castration. With two exceptions, the tissues were all predominantly basophilic in staining reaction,[4] and the depth of the endometrium

TABLE 1. ENDOMETRIAL TISSUES FROM CASTRATED AND UNTREATED MACAQUES

Group	Animal	Date of biopsy	Period of castration	Type of animal when castrated	Weight	Day of cycle	Ovarian structures
			days		kg.		
A	M.8	6/9	21	Cyclic adolescent	3.5	21	Follicles
	M.5	6/10	21	Acyclic	4.5	—	Follicles
	M.11	7/13	28	Cyclic mature	4.9	21	Follicles
	M.6	7/5	42	Cyclic mature	4.8	17	Follicles
B	M.12	7/12	28	Cyclic mature	3.6	20	Corpus and follicles
	M.10	8/1	35	Cyclic adolescent	4.8	17	Corpus and follicles
	M.4	7/4	42	Cyclic mature	4.5	25	Lutein tissue?

from myometrium to uterine lumen was shallow. The number of glands varied, some specimens containing a few straight ones, others containing many cross sections of glands. The gland epithelium was uniformly pseudostratified, but the nuclei varied in number and in size. They were usually crowded closely together, and were located at a uniform distance from the basement membrane. A few nuclei were observed in the basal ends of the cells, and these usually were irregular in outline, resembling pictures of nuclei in amitotic division. The stroma was composed of masses of closely packed nuclei. In each specimen there was an area of variable width and location in which these nuclei were much more densely packed than elsewhere in the stroma of that tissue (Plate 1, fig. 9, 10).[5] In some tissues this denser area lay immediately beneath the surface epithelium, in others, it lay beneath a superficial area of less compactly arranged nuclei. This region of closely packed nuclei in the stroma of endometrium from castrated untreated macaques was characteristic for all of the specimens in the group and did not occur in the endometrium of any of the castrated macaques which were treated with estrogen, progesterone, or the two in combination.

Cell characteristics of endometrium from castrated macaques whose ovaries did not contain lutein tissue at the time of castration. The epithelial cells were pale in their staining reaction, and varied in height in the different tissues and in the same tissue. The cell membranes were demonstrable as slender lines, delicately stained. The cytoplasm consisted of a clear, colorless hyaloplasm and a spongioplastic net. The hyaloplasm seemed to offer no obstruction to the passage of the transmitted light, and the strands of the spongioplasm took little or none of either the acid or basic stain (Plate 3, fig. 33). A few cells contained areas of clear hyaloplasm in which there was no spongioplastic net. The cytoplasm of the cells of the basement membrane was ill-

[4] In one specimen the chromatin of both epithelial and stromal nuclei was red, and in two other specimens some of the gland epithelial nuclei had acidophilic chromatin.

[5] The author is grateful to Dr. Mary P. Gray for the photographic work, and to Horace Cluxton for his assistance in securing the biopsy specimens.

defined, and the nuclei, which were numerous and closely spaced about many of the glands, were round or oval in form, and contained granular chromatin. The lumen surface of the cells was usually smooth in appearance. The nuclei had a chromatin network, between the meshes of which the clear colorless nucleoplasm was visible. The nucleoli were red-purple, black, or clear with a rim of black chromatin.

(*a*) *Stromal cells.* The stromal nuclei usually had no demonstrable cell cytoplasm or cell membrane. Between many of them a material was visible which did not stain well, and did not appear to offer obstruction to the transmission of light. In the deeper layers, the nuclei were more widely spaced, and the intercellular material which was observed in this region stained palely acidophilic. The nuclei had coarsely granular chromatin in a clear nucleoplasm, and nucleoli which were red, yellow or black.

(*b*) *Vascular bed.* The endothelium had an abundance of large pale staining nuclei with a chromatin network and a clear or faintly basophilic nucleoplasm. Many small endothelial vessels were patent in the region of the densely packed nuclei. The cytoplasm of the endothelium was pale and scarcely demonstrable. The arterioles were characterized by acidophilic hyalinization of the cytoplasm of the smooth muscle cells. This phenomenon appeared to have some association with the period elapsing between the time of castration and the time of biopsy. This hyalinization of the smooth muscle cells of the arterioles was characteristically seen in tissues from castrated untreated macaques, and was not seen in any of the tissues from animals injected with the hormones.

Cell characteristics of endometrium from castrated and untreated macaques whose ovaries contained lutein tissue at the time of castration (Plate 1, fig. 1; Plate 3, fig. 34). The gland cells of these tissues had a cytoplasm consisting of a colorless hyaloplasm and a spongioplastic net which was acidophilic in staining reaction. The hyaloplasm was glairy in character, appearing to offer some obstruction to the passage of the transmitted light. The spongioplastic net was more abundant in these cells, the material comprising it seeming to be composed of a denser material. In some cells there were areas of clear hyaloplasm between the row of nuclei and the basement membrane. The cytoplasm of these cells was usually more abundant than that of the cells in the first type of endometrium from castrated untreated macaques. The cell membranes were demonstrable, and the lumen surface of the cell membranes was smooth and straight in some cells, and irregular in contour in others. The nuclei were large, elongated and oval, pale staining, and usually basophilic in staining reaction, though this was not uniformly the case. The chromatin network was delicate, often wide-meshed, and the nucleoplasm visible between its strands had a glairy appearance. The nucleoli varied in appearance, being black, red, red-purple, pink, or colorless and surrounded by a rim of black chromatic material.

(*a*) *Stromal cells.* Outside of the area of densely packed stromal nuclei, the cytoplasm of many of the stromal cells was demonstrable. It was amphophilic and dense, obscuring the hyaloplasm, with the exception of a peri-nuclear area which was colorless and often glairy in appearance. The intercellular spaces contained demonstrable material which was acidophilic in staining reaction, and appeared to obstruct the passage of the transmitted light. The nuclei were large, and had a chromatin network between the meshes of which a glairy nucleoplasm was visible. The nucleoli were not prominent, and were red, black, or red-purple (Plate 1, fig. 9).

(*b*) *Vascular bed.* The endothelium had an abundance of nuclei with granular chromatin, and a demonstrable cytoplasm which was dense, and pale in staining reaction. The arterioles contained smooth muscle cells with hyalinized cytoplasm (Plate 2, fig. 17; Plate 2b, fig. 25).

Endometrium from Castrated Macaques Treated with Estrogen

The specimens of tissue in this group were from castrated macaques which had received a uniform dosage of 100 R.U. estrogen daily for periods of 2, 3, 4, 5 and 6 weeks (table 2).

TABLE 2. ENDOMETRIAL TISSUES FROM CASTRATED MACAQUES RECEIVING ESTROGEN

Animal	Date of biopsy	Treatment	Dosage per day	Total dosage
		days	R.U.	R.U.
M.1	5/4			
M.2	5/26	14	100	1400
M.12	7/27			
M.11	7/28			
M.8	6/30			
M.9	7/25	21	100	2100
M.7	6/18	28	100	2800
M.3	6/11			
M.8	7/14	35	100	3500
M.9	8/8			
M.7	7/2	42	100	4200
M.3	6/25			

These tissues will be described as a unit, since there were no apparent differences in cytoplasmic structure which could be attributed to the condition of the tissue at the time the injections of estrogen were begun, or to the period elapsing between the time of ovariectomy and the first injection of the hormone.

General tissue characteristics. With a few exceptions, all of the specimens of tissue from castrated macaques treated with estrogen, reacted strongly to both the basic

Plate 1. Fig. 1–8. GLAND EPITHELIUM FROM THE ENDOMETRIUM OF CASTRATED MACAQUES UNDER VARIOUS EXPERIMENTAL CONDITIONS. ×666

Fig. 1. M.12 7/12. Castrated 28 days previously.

Fig. 2. M.4 7/1. Castrated 42 days previously.

Fig. 3. M.12 7/27. Fourteen days estrogen, 100 R.U. per day.

Fig. 4. M.7 7/2. Forty-two days estrogen, 100 R.U. per day.

Fig. 5. M.10 8/23. Twenty-one days progesterone alone, 1 RB.U. per day.

Fig. 6. M.11 8/4. Estrogen priming, 14 days, 100 R.U. per day, followed by the same level of estrogen and 1 RB.U. progesterone per day for 7 days.

Fig. 7. M.6 8/3. Estrogen priming, 14 days, 100 R.U. per day, followed by the same level of estrogen and 1 RB.U. progesterone per day for 14 days.

Fig. 8. M.14 1/30. Estrogen priming, 14 days, total of 2700 R.U. estrogen, dropping to 15 R.U. per day on day 15 and continuing at that level for the next 13 days during which time a total of 37 RB.U progesterone were given. 3 RB.U. per day beginning on day 17.

Fig. 9–16. AREAS OF THE STROMA FROM THE ENDOMETRIUM OF CASTRATED MACAQUES UNDER VARIOUS EXPERIMENTAL CONDITIONS. ×666

Fig. 9. M.12 7/12. Castrated 28 days previously. Lutein tissue in the ovaries at the times of castration. Note dense area of stroma which lies beneath a less dense area. Surface epithelium at the top.

Fig. 10. M.4 7/1. Castrated 42 days previously. Narrow area of dense nuclei lies immediately beneath the surface epithelium in this specimen.

Fig. 11. M.12 7/27. Fourteen days estrogen, 100 R.U. per day. Cells dispersed, with strands of material traversing the intercellular spaces.

Fig. 12. M.7 7/2. Forty-two days estrogen, 100 R.U. per day.

Fig. 13. M.10 8/23. Twenty-one days progesterone alone, 1 RB.U. per day. Like M.12, this animal had lutein tissue in the ovaries at the time of castration. Cells dispersed, and intercellular material visible.

Fig. 14. M.11 8/4. Estrogen priming, 14 days, 100 R.U. per day, followed by the same level of estrogen and 1 RB.U. progesterone per day for 7 days. Intercellular spaces are not always empty as in this photograph. Cytoplasmic strands more opaque.

Fig. 15. M.6 8/3. Estrogen priming for 14 days, 100 R.U. per day, followed by the same level of estrogen and 1 RB.U. progesterone per day for 14 days. Increased amount of demonstrable cytoplasm.

Fig. 16. M.14 1/30. Estrogen priming, 14 days, total of 2700 R.U., level then dropped to 15 R.U. per day for 14 days, during which time a total of 37 RB.U progesterone were given.

All tissues were promptly fixed in Bouin's fluid, stained with hematoxylin and eosin, and cut at 5 μ. The photographs were made on Wratten plates, using K3 and daylight filters, 12.5× compensating ocular, 61× and 100× apochromatic oil immersion objectives.

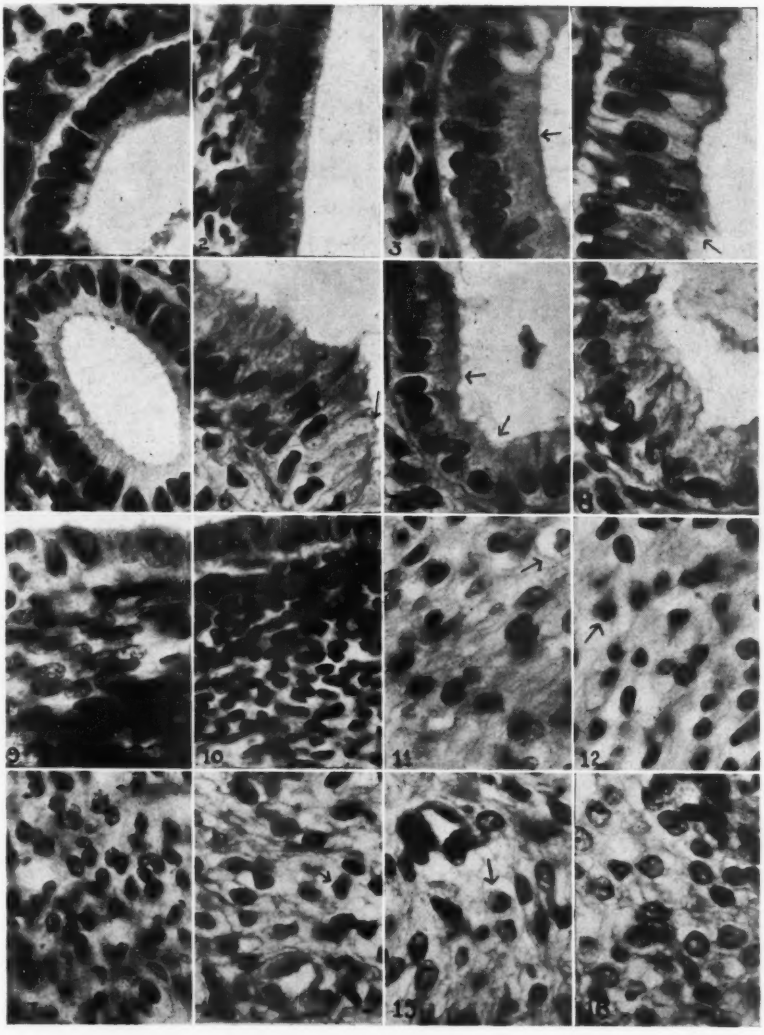

Plate 1. (See opposite page for legend.)

and acid stains. The depth of the tissue from myometrium to surface epithelium was greatly increased over that of the tissues from castrated untreated macaques.

The glands of the endometrium of castrates responded to the presence of estrogen by an apparent increase in their number, and an absolute increase in their length, since the depth of the endometrium could be as much as 8 times that of endometrium in castrated untreated macaques. The gland epithelium was pseudostratified in all of these tissues (Plate 1, fig. 3, 4), the degree of pseudostratification varying in different glands, and even in different parts of the same gland. Some glands were straight, others were twisted or coiled; some had a narrow lumen, others had a somewhat di-

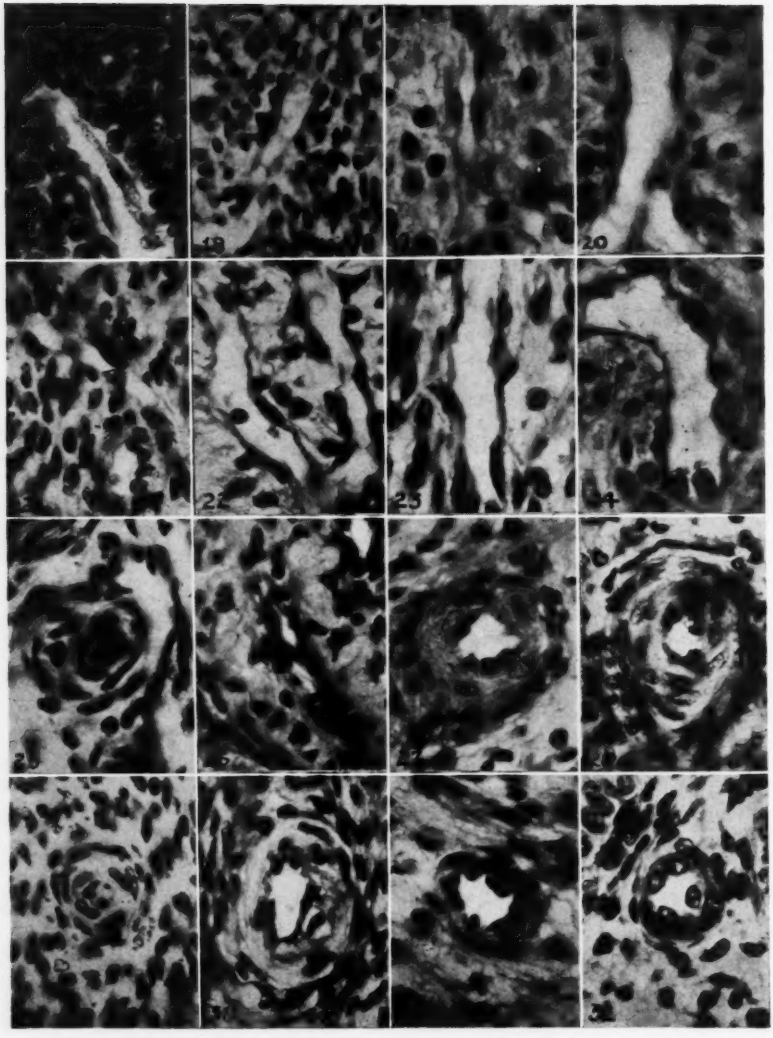

Plate 2. Fig. 17–24. ENDOTHELIUM IN THE STROMA OF ENDOMETRIUM FROM CASTRATED MACAQUES UNDER EXPERIMENTAL CONDITIONS. ×666

Fig. 17. M.12 7/12. Castrated 28 days previously. Capillary lying in the dense area of the stroma.

Fig. 18. M.4 7/1. Castrated 42 days previously.

Fig. 19. M.12 7/27. Fourteen days estrogen, 100 R.U. per day. Cytoplasm is not great in amount and not dense in staining reaction.

Fig. 20. M.7 7/2 Forty-two days estrogen, 100 R.U. per day.

Fig. 21. M.10 8/23. Twenty-one days progesterone alone, 1 RB.U. per day. Large nuclei in the endothelium.

Fig. 22. M.11 8/4. Estrogen priming for 14 days, 100 R.U. per day, followed by the same level of estrogen and 1 RB.U. progesterone per day for 7 days. Endothelial cytoplasm more sharply defined.

Fig. 23. M.6 8/3. Estrogen priming 14 days, 100 R.U. per day, followed by the same level of estrogen and 1 RB.U. progesterone per day for 14 days.

(*Continued on next page*)

lated lumen. There was no apparent correlation between the degree of pseudostratification and the form of the glands, or the degree of glandular dilatation. The nuclei of the gland cells were not uniformly located at the same level in the cells, as was observed in the glands of tissues from castrated untreated macaques. Some nuclei lay in the more distal portion of the tall epithelial cells, others were more basally placed, and others were centrally located (Plate 3, fig. 35). Mitotic figures were abundant and uniformly appeared in the distal end of the cell not far from the lumen surface of the cell membrane (Plate 1, fig. 3). This position and the great number of the mitotic figures contrasted with the basal location of probable amitotic figures in the endometrium from castrated untreated animals.

The stroma characteristically reacted to the presence of estrogen by a dispersion of the stromal nuclei, the appearance of a demonstrable cell membrane and cytoplasm, and the appearance of intercellular spaces, some of which contained demonstrable acidophilic material (Plate 1, fig. 11, 12).

Cellular characteristics. (*a*) *Gland cells.* The cell membranes, distal, lateral and basal, of the tall pseudostratified epithelium were well defined in the tissues of castrated animals treated with estrogen. The cytoplasm of these cells, usually acidophilic, contained a demonstrable spongioplasm, and a hyaloplasm which varied in appearance (Plate 1, fig. 3, 4). The spongioplasm consisted of two reticular nets. The first, and more easily discernible, of these reticular structures was composed of a network of fine fibers, the meshes of which were small in some cells, larger in others; sometimes this variation in appearance appeared to be associated with the size of the cell, while the density of the staining reaction frequently seemed to be associated with the size of the cell and the compactness of this first spongioplastic net. A few clumps of acidophilic material appeared to lie within or upon the fibers of the network. The second spongioplastic net was usually more centrally located in the cells. It was composed of a few broad acidophilic, refractile fibers with relatively wide areas of clear hyaloplasm between the fibers (Plate 1, fig. 3, 4, arrow; Plate 3, fig. 35, arrows).

The hyaloplasm in the epithelial cells was usually colorless and transparent. A few low pseudostratified epithelial cells appearing to have a dense homogeneous strongly acidophilic cytoplasm were found to have both spongioplasm and hyaloplasm which were acidophilic in staining reaction.

In the immediate supranuclear position of a few cells there was an area of differentiated cytoplasm which corresponded in location to that of the negative image of the Golgi apparatus. These areas were characterized by the presence of two or more small pieces of refractile acidophilic material with narrow areas of a clear glairy cytoplasm surrounding and passing between them.

Fig. 24. *M.14* 1/30. Estrogen priming for 14 days, total of 2700 R.U. estrogen, dropping to 15 R.U. estrogen per day for 14 days, with simultaneous administration of progesterone, total of 37 RB.U.

Fig. 25–32. ARTERIOLES IN THE ENDOMETRIUM OF CASTRATED MACAQUES UNDER VARIOUS EXPERIMENTAL CONDITIONS. It was not possible to obtain photographs of vessels of comparable size in all of the different specimens. ×666

Fig. 25. *M.12* 7/12. Castrated 28 days previously. Arterioles showing some hyalinization of the cytoplasm of the smooth muscle cells. Cross-section.

Fig. 26. *M.4* 7/1. Castrated 42 days previously. Longitudinal section of arteriole showing nuclei in midst of hyalinized acidophilic cytoplasm of the smooth muscle cells.

Fig. 27. *M.12* 7/27. Fourteen days estrogen, 100 R.U. per day. Cross-section. Demonstrable cytoplasm of smooth muscle cells.

Fig. 28. *M.7* 7/2. Forty-two days estrogen, 100 R.U. per day. Cross-section of arteriole.

Fig. 29. *M.10* 8/23. Twenty-one days progesterone alone, 1 RB.U. per day.

Fig. 30. *M.11* 8/4. Estrogen priming, 14 days, 100 R.U. per day, followed by that same level of estrogen and 1 RB.U. progesterone per day for 7 days.

Fig. 31. *M.6* 8/3. Estrogen priming 14 days, 100 R.U. per day, followed by the same level of estrogen and 1 RB.U. progesterone per day for 14 days.

Fig. 32. *M.14* 1/30. Estrogen priming for 14 days, total of 2700 R.U., dropping to 15 R.U. per day for the succeeding 14 days during which time a total of 37 RB.U. progesterone were administered. Note density of cytoplasm of the smooth muscle cells.

In some of the epithelial cells there was a narrow zone of modified hyaloplasm immediately surrounding the nucleus. This area was glairy in appearance, resembling that colorless area seen in the region of the negative image of the Golgi net. When the highest magnification and optimum lighting conditions available were used, and when the focus on the section was gradually changed, strands of chromatic material in the nucleus appeared to extend beyond the apparent nuclear membrane into this narrow area of modified hyaloplasm.

In the region of the epithelial cells lying below the nuclei there were clear vacuoles. These were found in some glands, in every specimen of endometrium secured from castrated animals which had been treated with estrogen. The size of these vacuoles varied from a small, relatively inconspicuous structure to a large vacuole which pushed the nucleus far into the distal region of the cell. Often the clear vacuole occupied the entire area of the cell between the nucleus and the basal surface of the cell. Rod cells occurred among the gland epithelial cells. The gland lumen surface of the epithelial cells presented a variety of appearances, some cells being ciliated, others having a smooth and intact surface membrane. Homogeneous acidophilic masses of material in the gland lumen often lay in juxtaposition to the surface of the gland cells. Small oval acidophilic structures were observed in the luman of some of the glands.

The basement membrane of the glands was well defined and often wide (Plate 1, fig. 3, 4). The nuclei of the membrane were basophilic, with granular chromatin and the cytoplasm was acidophilic and variable in amount.

The nuclei of the gland epithelial cells were characterized by a thick nuclear membrane, a coarse chromatin network, large nucleoli, and a clear nucleoplasm. All of these characteristics appeared to be intensified the longer the period of treatment with estrogen. The prominent nucleoli were pink, blue, red, purple and black in the different nuclei (Plate 3, fig. 35).

(*b*) *Stromal cells.* When estrogen had been administered to castrated macaques, there was no zone of closely packed basophilic nuclei in the superficial area of the stoma, the nuclei being comparatively widely separated (Plate 1, fig. 11, 12). There was demonstrable acidophilic intercellular material in many of these tissue spaces, especially in a narrow area lying immediately beneath the surface epithelium.

The stromal cells were stellate and spindle in form, some of the cell processes being quite long. The cell membranes were well defined, and in most of the cells a spongioplastic net of fine acidophilic interlacing fibers were visible. There were a few round or polygonal cells in the stroma which had well defined cell membranes, a colorless and often glairy hyaloplasm, and a spongioplasm in the form of a cluster of refractile acidophilic granules in the region of the nucleus (Plate 1, fig. 11, 12, arrow).

The nuclei of the stromal cells had the same general characteristics as those of the nuclei in the epithelial cells, i.e., thick nuclear membrane, coarse strands of chromatin, prominent nucleoli, and clear nucleoplasm. The nuclei of the round or polygonal cells which contained the acidophilic granules, frequently stained more heavily with hematoxylin than the nuclei of the stellate or spindle cells.

Vascular tissue characteristics. The arterioles did not show any evidence of hyalinization of smooth muscle cells such as was seen in the walls of arterioles in specimens of endometrium from castrated and untreated macaques (Plate 2, fig. 27, 28). The endothelial nuclei were usually large, well stained structures with granular chromatin, a faintly basophilic nucleoplasm, and a well defined nuclear membrane. Few or no spiral arterioles or capillaries with occluded lumen were observed in the tissues from castrated animals treated with estrogen.

The more superficial areas of the stroma were traversed by strands of endothelium about which no smooth muscle cells or modified stromal cells appeared. The majority of these patent endothelial tubes had walls which were composed of many endothelial cells with basophilic, aggregated nuclei and a small amount of pale acidophilic cytoplasm (Plate 2, fig. 19, 20). Mitotic figures in the endothelial cells were not an uncommon occurrence.

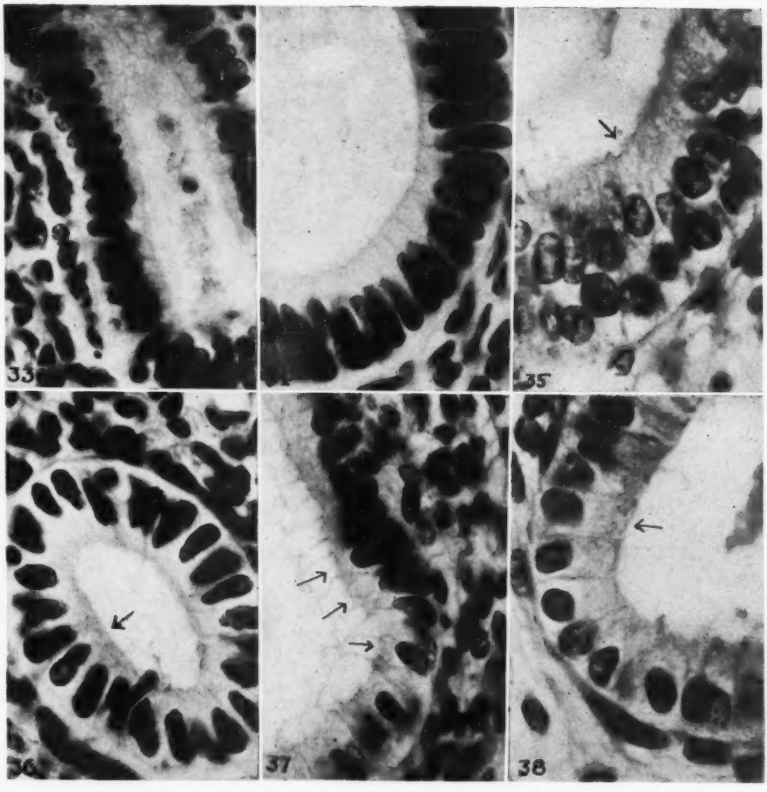

Plate 3. GLAND EPITHELIUM FROM THE ENDOMETRIUM OF CASTRATED MACAQUES UNDER VARIOUS EXPERIMENTAL CONDITIONS. ×1000

Fig. 33. M.5 6/10. Castrated 21 days previously. Animal acyclic prior to castration. No lutein tissue in ovaries. Network in cytoplasm demonstrable, but pale staining.

Fig. 34. 7/12. M.12. Castrated 28 days previously. Lutein tissue in ovaries when castrated. Cytoplasm more acidophilic and more easily demonstrable.

Fig. 35. 7/2. M.7. Forty-two days estrogen, 100 R.U. per day. Note height of cells, distribution of nuclei at different levels, aggregate chromatin in nuclei, well-defined spongioplastic networks, and subnuclear vacuoles.

Fig. 36. M.5 7/1. Twenty-one days progesterone alone. Same animal as that in Fig. 33. Cytoplasm poorly defined.

Fig. 37. M.10 8/23. Twenty-one days progesterone alone. The animal was castrated for 35 days prior to giving the progesterone, and there was lutein tissue in the ovaries when they were removed. Fig. 36 and 37 are from castrated animals receiving identical treatment. The difference in tissue response probably depends on the difference in the nature of the tissue in the castrated animals.

Fig. 38. M.6 8/3. Estrogen priming for 14 days, 100 R.U. per day, followed by the same level of estrogen and 1 RB.U. progesterone per day for the next 14 days.

Endometrium From Castrated Macaques Receiving Progesterone Alone

The two specimens of tissue in this group were obtained from castrated macaques treated for 21 days with 1 RB.U. progesterone per day (table 3).

General tissue characteristics. The histologic characteristics of endometrium from castrated and untreated macaques were not greatly modified by the action of pro-

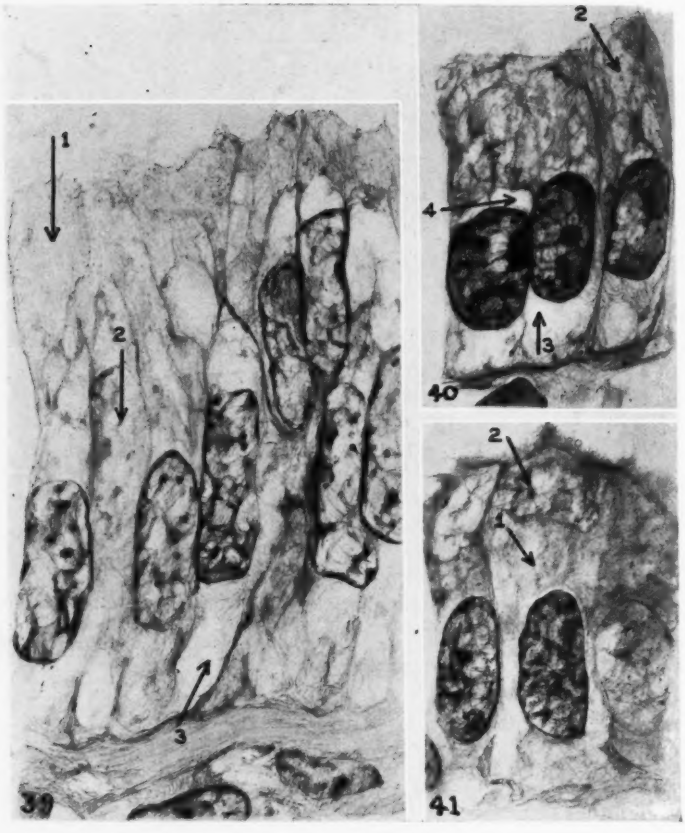

Plate 4. PHOTOGRAPH OF CAMERA LUCIDA DRAWINGS OF ENDOMETRIUM FROM CASTRATED MACAQUES TREATED WITH ESTROGEN AND PROGESTERONE. All drawings made at the same magnification by Susan Wilkes.

Arrow 1, primary spongioplastic net; Arrow 2, secondary spongioplastic net; Arrow 3, subnuclear vacuole; Arrow 4, perinuclear clear area.

Fig. 39. M.12 7/27. Fourteen days estrogen, 100 R.U. per day. Fig. 40. M.7 7/2. Forty-two days estrogen, 100 R.U. per day. Fig. 41. M.4 12/7. Fourteen days estrogen, 100 R.U. per day, followed by the same level of estrogen and 2 RB.U. progesterone per day for 14 additional days.

gesterone alone (Plate 1, cf. fig. 1, 5; Plate 1, fig. 9, 13; Plate 2, fig. 17, 21; Plate 2, fig. 25, 29). The endometrium was somewhat deeper from myometrium to lumen surface, following treatment with the progesterone, and the staining reaction of the intercellular elements and the cytoplasm was more acidophilic. In some glands there were nuclei in the basal ends of the cells resembling those described as undergoing amitotic division (Plate 1, fig. 5). The cell membranes were defined, and the basement membrane was demonstrable with slender elongated nuclei.

The stroma was less densely packed with nuclei than in endometrium from castrated and untreated animals, the intercellular material was more abundant, and the cytoplasm of the stromal cells was demonstrable (Plate 1, cf. fig. 9, 13).

Beyond these general characteristics, the two tissues had little in common, for the cellular characteristics varied as greatly as the cellular characteristics in the

endometrium of the control specimens. The animals used for this part of the experiment fundamentally differed in that one of them had lutein tissue in the ovary at the time of castration, and the other one did not.

Cell characteristics of the endometrium of a castrated macaque treated with progesterone (no lutein tissue in the ovary at the time of castration). The epithelium of the glands was simple in many places, and somewhat pseudostratified in others (Plate 3, fig. 36). The cells were tall, some of them being wide, others narrow. Rod cells occurred in several glands. The cell membranes were demonstrable, and the nuclei usually were located at a uniform distance from the basement membrane. A single type of spongioplastic net was observed. It was palely acidophilic and composed of a

TABLE 3. ENDOMETRIAL TISSUES FROM CASTRATED MACAQUES RECEIVING PROGESTERONE

Animal	Date of biopsy	Treatment	Dosage	Total dosage
		days	RB.U.	RB.U.
M.5	7/1	21	1	21
M.10	8/23	21	1	21

few broad strands of material which were more widely spaced in some cells, and more compactly arranged in others. The colorless hyaloplasm had a slightly glairy appearance, as though the light were diffused in transmission. An area of clear hyaloplasm was often seen in the supra-nuclear, as well as in the peri-nuclear region of the epithelial cells (Plate 3, fig. 36, arrow). The elongated nuclei had a close-meshed chromatin network in a glairy basophilic nucleoplasm.

(*a*) *Stromal cells.* In the superficial area the stromal nuclei were more dispersed than they were in the superficial area of the stroma prior to treatment with progesterone alone. However, the small nuclei were much closer together than in the superficial stroma of endometrium from castrated macaques treated with estrogen. As a whole, the stroma presented a uniformly glairy appearance, and neither cell membranes, cytoplasm nor intercellular material reacted with the acid or basic stain. A few of the stromal nuclei had an area of clear hyaloplasm surrounding the nucleus. Some cells were long and spindle shaped with demonstrable cytoplasm. The stomal nuclei were smaller than those of the epithelium, but otherwise they morphologically resembled them.

(*b*) *Vascular bed.* The endothelial cells were numerous and they had a cytoplasm which was amphophilic and opaque in character. The cytoplasm of the large smooth muscle cells of the arterioles contained a few strands of pale acidophilic spongioplasm in a glairy hyaloplasm. There was no evidence of hyalinization of the arterioles such as was observed in the endometrium of castrated and untreated macaques. The endothelial cells varied in appearance, some having nuclei which were small and heavily stained in some vessels, others being large pale structures.

Cell characteristics of the endometrium of a castrated macaque treated with progesterone alone (lutein tissue present in the ovary at the time of castration). The appearance of the gland cells varied greatly in the different glands of this tissue. The photograph on plate 3, figure 37 shows a single gland in which the cells presented a variety of appearances. The cells varied in height, some being very tall, others, low. The tall cells were usually narrow though some of them were wide. The cell membranes were well defined, and the cytoplasm consisted of two demonstrable spongioplastic nets and a colorless hyaloplasm. The two nets resembled those described in the gland cells of specimens of endometrium from castrated animals treated with estrogen alone. The networks were acidophilic, and the density of the first one seemed to vary with the width and the height of the cell. The nuclei in many glands were abundant,

long and narrow; they had a chromatin net of medium width lying in a faintly acid nucleoplasm. Clumps of chromatic material appeared in the cytoplasm of many of the cells, and many of the nuclei were bizarre in form.

(*a*) *Stromal cells.* There was no region of densely packed nuclei in the stroma of this tissue. The superficial area immediately beneath the surface epithelium contained a few widely spaced compressed cells with a dense acidophilic cytoplasm. The intercellular spaces in this region were filled with oval structures consisting of a central colorless area and an acidophilic limiting surface. The structures were packed closely together in honey-comb fashion, and they may have represented closely packed red blood cells. In the deeper areas of the stroma the cells had more cytoplasm in which a few strands of well-stained spongioplasm and a peri-

TABLE 4. ENDOMETRIAL TISSUES FROM CASTRATED MACAQUES PRIMED WITH ESTROGEN AND TREATED WITH ESTROGEN AND PROGESTERONE IN COMBINATION

Group	Animal	Date of biopsy	Estrogen priming		Combined estrogen and progesterone		
			Days	Dosage per day	Days	Estrogen per day	Progesterone per day
				R.U.		R.U.	Rb.U.
A	M.2 M.12 M.11	7/2 8/3 8/4	14	100	7	100	1
B	M.4 M.6	8/2 8/3	14	100	14	100	1
C	M.10 M.9 M.4	12/5 12/6 12/7	14	100	14	100	2
D	M.9 M.14	1/30 1/30	14	*	14	15	3

* Estrogen, 100 R.U. per day for 10 days, 200 R.U. on day 11, and 500 R.U. per day for each of the following 3 days.

nuclear clear area of hyaloplasm were visible. The extensive processes of these polygonal cells were broad, acidophilic, and often refractile (Plate 1, fig. 13).

(*b*) *Vascular bed.* The large endothelial cells had well-stained cytoplasm and nuclei containing a chromatin net in a clear nucleoplasm. The smooth muscle cells of the arterioles had large nuclei and pale cytoplasm consisting of colorless glairy hyaloplasm and strands of pale spongioplasm (Plate 2, fig. 21; Plate 2, fig. 29).

Endometrium of Castrated Macaques Treated with Estrogen and Progesterone

The material available for study in this group of tissues was limited since none of the specimens of endometrium were obtained on the first day of bleeding, and, with but two exceptions, all of the specimens were from animals which received the same daily dosage of estrogen during the period of treatment with progesterone as during the period of priming with the estrogen. Table 4 gives a summary of the experimental procedures for the animals treated with both estrogen and progesterone.

General tissue characteristics. Histologically, endometrium primed with estrogen responded to the presence of progesterone by a change in the staining reaction of the tissue in general, an increase in the amount of the cytoplasm of the cells, and a modification of the distribution of granular chromatin in the nuclei. The glands were widely dilated and the degree of dilatation and serration appeared to be roughly pro-

portional to the amount of progesterone administered, and to the length of time for which it was given. The tissue spaces in the stroma appeared to be larger than in tissue from animals treated with estrogen alone, and cytoplasmic strands connecting the various stromal cells traversed many of these spaces (Plate 1, fig. 14, 15, 16).

Tissues in group A. (Table 4.) The tissues in this group were from animals primed for 14 days with 100 R.U. estrogen per day, and subsequently treated with the same amount of estrogen to which was added 1 RB.U. progesterone per day for 7 days.

The tissues in this group could be described as manifesting some progestational modifications, but these were not extensive when the tissues were compared with specimens from animals receiving larger amounts of progesterone.

Cell characteristics. (*a*) *Gland cells* (Plate 1, fig. 6.) The gland epithelium in the presence of a small amount of progesterone acting for a short period of time showed little more than a tendency to become the simple type of epithelium. The cell membranes were defined, but not as sharply as in tissues from animals receiving only estrogen. The hyaloplasm had undergone a change difficult to define, since it was not as clear and transparent as that characteristic for the epithelial cells of endometrium from animals receiving estrogen alone. The hyaloplasm had the appearance of being slightly glairy, and more dense. This change in the hyaloplasm may have been responsible in part for the modified appearance of the first spongioplastic net which was less sharply defined, and was paler in its staining reaction than that usually seen in tissues from animals receiving estrogen alone. This net was composed of fine delicate fibers, and at many points within or upon it there were small clumps of cytoplasmic material. The second reticular network with its broad strands was also pale staining, and usually appeared to be less sharply defined than in tissues from animals treated with estrogen alone (Plate 1, fig. 6, arrow).

No structures which could be construed as the negative image of the Golgi apparatus were observed in the epithelial cells of these tissues. Surrounding many of the epithelial nuclei were wide areas of glairy hyaloplasm, and many of the nuclei were distorted and irregular in contour. Assuming that the mode of fixation was responsible for the appearance of the nuclei, it was interesting that the distortion was not observed in tissues following treatment with estrogen alone, and it is probable that the physiologic state of the tissue resulting from the presence of progesterone was responsible for the slow rate of penetration of the fixative since the technic of preparing the tissues was uniform throughout the experiments.

In contrast to the great number and sharp definition of the subnuclear vacuoles in tissues from animals receiving estrogen only, there were but few subnuclear vacuoles in the gland cells following treatment with progesterone, and these were usually traversed by relatively coarse strands of spongioplasm.

Cells resembling those described as 'rod' cells in human endometrium were found in tissues from animals receiving both estrogen and progesterone. Such structures were comparatively few in tissues from animals receiving estrogen alone. Usually the cytoplasm of these rod cells appeared to be more acidophilic than that of adjacent cells.

The lumen surface of the gland cells in this group of tissues was generally uneven. In some areas strands of cytoplasmic material, acidophilic in character, extended into the gland lumen. Other cells had a smooth lumen surface. The height of the epithelium varied, which was probably the result of secretory activity. The basement membrane was well defined, its width varying in different areas, though it was often slender, with numerous compressed heavily stained nuclei and an opaque dense cytoplasm of amphophilic staining reaction.

The nuclei of the epithelial cells were characterized by a thin nuclear membrane within which lay a delicate, thin, close-meshed network with clumps of chromatin lying at many of the intersections of the net. The nucleoplasm was obscured to some extent by the chromatin network, but apparently it lacked the clarity of the nucleoplasm observed in the nuclei of tissues from animals receiving estrogen only. The chromatin was abundant, and the small nucleoli were black or purple in color.

(*b*) *Stromal cells.* (Plate 1, fig. 14). The cell walls and cytoplasm of the stromal cells appeared to have undergone the same type of modification as that described for the epithelial

cells. The amount of cytoplasm was greater, the staining reaction was paler and the cell membranes and spongioplasm were less sharply defined than in tissues from animals receiving estrogen only. The tissue spaces, some of which contained demonstrable material, were traversed by many strands which were probably processes of the stellate cells. There were many round or polygonal cells with a well defined cell membrane, a clear, and sometimes a glairy, hyaloplasm containing a clump of refractile acidophilic granules in juxtaposition to the centrally located nucleus (Plate 1, fig. 14, arrow).

In some areas the stromal cells were compressed between the dilated glands; they appeared to be spindle shaped cells with densely stained nuclei and cytoplasm.

Some of the nuclei of the stromal cells resembled the nuclei of the epithelial cells because of their thin nuclear membrane and delicate close-meshed network. Other nuclei in the stromal cells resembled the nuclei uniformly found in tissues from animals receiving estrogen only, i.e., nuclei with thick nuclear membrane, and coarse strands of chromatin widely separated in a clear nucleoplasm. The nucleoli were pink in the nuclei of the stroma. Some nuclei with solid homogeneous nongranular chromatin were observed in the stroma of endometrium from animals receiving both estrogen and progesterone.

(*c*) *Vascular bed.* The outstanding modification of the vascular bed apparently produced by the presence of progesterone seemed to be in the cytoplasm of the endothelial cells (Plate 2, fig. 22). The cytoplasm usually stained a red-purple color, appeared to be opaque and homogeneous in character, and frequently was more abundant than it was in tissues from animals receiving estrogen alone. Some of the vessels were dilated to the point of resembling sinusoidal spaces and were composed of a single layer of endothelial cells with well-defined cytoplasm. A few smooth muscle cells were visible about some of these vessels. Some capillaries were small in diameter, and had but one nucleus per cross-section. A few endothelial tubes with two and three layers of nuclei were observed in these tissues. The nuclei were often large and smooth in contour, giving no evidence of shrinkage. Cross-sections of the arterioles revealed little modification from the structures observed in the tissues from animals receiving estrogen alone, other than that the cytoplasm of the smooth muscle cells had a spongioplasm which was dense and opaque.

Tissues in group B. (Table 4.) The tissues in this group were from animals primed for 14 days with 100 R.U. estrogen per day and subsequently treated with the same amount of estrogen to which was added 1 RB.U. progesterone per day for 14 additional days.

Tissue characteristics. The glands in this group of tissues were well dilated, and for the most part were composed of simple epithelium, some of which was columnar, much of which was low and wide in form. The lumen surface of the gland epithelium was often irregular in contour, but no real bleb-like protrusions were observed. Masses of acidophilic material filled the lumen of some of the glands.

Cellular characteristics. (*a*) *Gland cells.* As in the tissues of *group A*, the two spongioplastic networks in the epithelial cells were not sharply defined, and they varied from acid to basic in staining reaction (Plate 1, fig. 7; Plate 3, fig. 38). Small clumps of material often appeared at intervals in the first fine meshed non-refractile network. Some of the cells had cytoplasm which appeared to be opaque, but study with oil immersion lens revealed that both hyaloplasm and spongioplasm were acidophilic. Generally the hyaloplasm was colorless. There were a few subnuclear vacuoles with strands of spongioplastic network crossing the surface. A wide area of clear glairy hyaloplasm surrounded many of the epithelial nuclei.

The nuclei varied in shape, some being round, and others elongated. The nuclear membrane was irregular in outline in some cells, but not in as many as in the tissues in *group A*. The chromatin of the nuclei was arranged in the same manner, but appeared to be paler in its staining reaction. The nucleoli were black, pink or purple, with a well defined rim of basic staining chromatin surrounding them.

(*b*) *Stroma.* (Plate 1, fig. 15.) Many of the tissue spaces in this group did not contain demonstrable material but they were traversed and separated by delicate fibers. When present, the intercellular substance was amorphous and acidophilic. Some of the stellate

stromal cells had ill-defined cell membranes and spongioplastic networks. There were many oval stromal cells (Plate 1, fig. 15, arrow) with a well defined cell membrane, clear or glairy hyaloplasm and a clump of acidophilic granules in the region of the nucleus, which was frequently shrunken and irregular in outline and deeply stained with hematoxylin.

(*c*) *Vascular bed.* There were small arterioles in the superficial area of the stroma, as well as some vascular tubes composed of many nuclei with dense staining chromatin and opaque amphophilic cytoplasm (Plate 2, fig. 31; Plate 2, fig. 23). In many of the larger capillaries the several nuclei were not uniformly distributed in the cytoplasm about the lumen of the vessel, but were grouped together on one side of the vessel, the other side being composed of an extensive area of well stained opaque cytoplasm in which no nuclei were visible. This characteristic was not confined to this group of tissues, however, since it occurred in some tissues from animals receiving estrogen alone.

Tissues in group C. (Table 4). The tissues in this group were from animals primed for 14 days with 100 R.U. estrogen per day and subsequently treated with the same amount of estrogen to which was added 2 RB.U. progesterone per day for 14 additional days.

General tissue characteristics. The general appearance of the tissues in this group was similar in many respects to that of the tissues in *group B.* As a whole, however, the staining reaction was paler, and there was a tendency toward tufting in the gland epithelium which was of the simple columnar type in most of the glands. In many cells the nucleus was rounded and basally located. In some cells the elongated nucleus was also basally located, but its long axis was perpendicular to the long axis of the columnar cell, rather than parallel to it as is usually the case. The nuclei of the gland epithelium were paler staining than the nuclei of the tissues in *group C*, table 4. The epithelial cells varied in height, staining reaction and state of preservation, glairy hyaloplasm containing poorly stained spongioplastic networks. Some of the glands were lined with simple cuboidal epithelium whose cytoplasm was acidophilic and appeared to be dense and opaque. This cytoplasm somewhat resembled some of the glands in tissues from animals receiving estrogen alone.

(*a*) *Stroma.* The cells of the stroma of these tissues were large and often had poorly defined cell membranes. The round cells with clear hyaloplasm and acidophilic granules lying near the nucleus were more numerous in these tissues than in any of the tissues described above. In addition there appeared a few long stromal cells, roughly triangular in shape which contained a dense well stained spongioplastic net within the cell membrane. The tissue spaces were more numerous than they were in the stroma of the tissues in *group A* and *B.*

Aside from these variations, the characteristics of the stroma in this group of tissues were much the same as those in *group A* and *B.*

(*b*) *Vascular bed.* The general characteristics of the vascular bed were similar to those for *group B.* However, the nuclei of the endothelium and its smooth muscle wall were hypertrophied, pale in their staining reaction, and had a clear nucleoplasm.

Tissues in group D. (Table 4.) The tissues in this group were from animals primed with estrogen for 14 days, the dosages being 100 R.U. per day for the first 10 days and increasing daily until 2700 R.U. had been given; on day 15 the dosage was dropped to 15 R.U. per day and maintained at this level for the succeeding 14 days during which time progesterone in increasing amounts to 3 RB.U. per day was administered.

There was a greater degree of gland dilatation in this group of tissues than in group *A*, *B*, or *C*. The gland epithelium was of the simple columnar and cuboidal type, with evidence of tufting in some of the glands although some glands had pseudostratified epithelium. The cell membranes were well defined, and often globules of material composed of a clear colorless central area, and an acidophilic surface were observed in juxtaposition to the lumen surface of the cells. The spongioplastic net in the

epithelial cells was amphophilic, and varied in density, being fine delicate and pale staining in some cells, coarse and more heavily stained in others. The second spongioplastic net, composed of more widely spaced refractile fibers, appeared to lie in the more distal area of the cell nearer to the lumen surface of the cell than was seen in any of the tissues described above (Plate 1, fig. 8).

Most of the simple cuboidal cells had rounded nuclei, and some of the simple columnar cells likewise. Many of the simple columnar and all of the pseudostratified epithelial cells had elongated nuclei. The delicate chromatin network was paler staining and apparently the chromatin was less abundant in the nuclei of this group of tissues than in any of those in the preceding groups. The nucleoplasm, though not greatly obscured by the pale delicate chromatin network, lacked the clarity and transparent quality of the nucleoplasm in tissues from animals receiving estrogen alone.

(*a*) *Stroma.* (Plate 1, fig. 16.) There was a wide, almost acellular area in the stroma immediately beneath the surface epithelium in the tissues of this group. Many of the extensive intercellular spaces contained a palely acidophilic material. In the deeper areas of the stroma there was an abundance of the round stromal cells with well defined cell membrane, clear hyaloplasm and a clump of acidophilic granules in the region of the nucleus. The nuclei of these cells were usually of irregular contour and heavily stained with hematoxylin. Stomal nuclei apparently undergoing amitotic and mitotic division were frequently observed.

(*b*) *Vascular bed.* (Plate 2, fig. 24; Plate 2, fig. 32). The elements of the vascular bed presented a great variety of appearances. In the wide acellular area of the stroma immediately beneath the surface epithelium, there were widely dilated vessels composed of a single layer of endothelium whose large nuclei had a thin well-stained nuclear membrane and a delicate chromatin network. The endothelial cytoplasm was abundant and dense. The smooth muscle cells of the arteriole walls were hypertrophied and the cytoplasm was dense, opaque and amphophilic in staining reaction.

The endothelial nuclei of some of the vessels in the stroma projected into the lumen of the vessel, and the endothelial nuclei contained densely stained chromatin and nucleoplasm. Other vessels of large irregular size had endothelial nuclei which did not project into the lumen of the vessel. In a few places strands of endothelial nuclei without notable cytoplasm or visible lumen were observed. Many leucocytes were present in the lumen of the dilated vessels.

DISCUSSION

The fact that the observations reported above were made on specimens of endometrium from animals in which the last injection of hormone material was made 24 hours prior to the time of biopsy, somewhat limits the usefulness of this study.

The hyaloplasm and nucleoplasm of fixed preparations of tissues from castrated animals under the influence of the corpus luteum factor appeared to transmit less light than these same areas in tissues from animals receiving estrogen alone. In tissues from animals treated with progesterone, the light seemed to be diffused in passing through the hyaloplasm and nucleoplasm, givin a mucoid or glairy appearance to these same regions. The clarity of the hyaloplasm and nucleoplasm under the influence of estrogen alone was striking. Since estrogen produces a swelling of the genital tissues generally, it may be that this cytologic clarity was due to the dilution of the cell constituents by the imbibed water. When progesterone was added to the experimental treatment, the amount of light transmitted through these structures in fixed preparations, appeared to be reduced. This was probably not the result of dehydration, since O'Leary (19), observed that the nuclear and cell volumes of human endometrial tissues were greater in the early progravid phase than in the proliferative phase. It is probable that this change in the character of light transmission under the influence of progesterone was the result of a physical or chemical alteration in the relationship between the imbibed water and the cell constituents.

SUMMARY

The cytologic and histologic structure of the endometrium of castrated macaques under various experimental conditions has been described. The observations on the histologic response of the endometrium of castrated macaques to hormonal stimuli have added little to the scattered reports already in the literature. However the cytology of the epithelial, connective and vascular tissues comprising the endometrium has not been previously described in specimens prepared by routine laboratory technic.

This study revealed a difference in the character of endometrial tissues from castrated and untreated macaques which, from the evidence at hand, appeared to depend upon the type of structure in the ovary at the time of castration.

The study likewise revealed that there were two spongioplastic networks in the cytoplasm of gland epithelial cells in the endometrium of castrated macaques under the influence of the ovarian hormones.

In castrated and untreated macaques the smooth muscle cells of the arterioles had undergone an acidophilic hyalinization that was not seen in the endometrium of castrated macaques treated with the ovarian hormones.

In tissues from animals treated with both estrogen and progesterone, the cytoplasm of the endothelium was more abundant, more dense and more heavily stained than it was in tissues from animals receiving estrogen alone.

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THE PREPARATION OF THYROTROPIC HORMONE[1]

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THE PRESENT KNOWLEDGE of the chemical nature of the pituitary hormones is inadequate. In view of the importance of the hypophysis in the control of the thyroid gland, the chief objective of the present study was to develop more satisfactory methods of extraction, separation and purification of the thyrotropic hormone. Methods of preparation and purification of this hormone have been reported (1–4). In the preliminary work we attempted to evaluate quantitatively the usefulness of each of these procedures. Our experience in this regard indicates that a large percentage of the thyrotropic hormone is discarded in these methods of purification.

Methods of biological assay. The analytical determination of the thyrotropic activity of the pituitary extracts was based upon the histological changes in the thyroid of the guinea pig according to the method of Junkmann and Schoeller (1) and in the thyroid of the 8 to 11-day-old White Leghorn chick. In the assay of our preparations, we have observed that the chick thyroid is usually more sensitive to the thyroid stimulating factor than the guinea pig thyroid. The chick must be of high quality and in excellent condition otherwise the death rate will cause considerable discouragement. In the interest of speed and economy of effort, preparations assayed on the chick were injected subcutaneously twice daily for 2 days and the birds killed 14 to 18 hours following the last injection. In general, our findings are in accord with those of Smelser (5) published since this work was completed, and need not be repeated here. Smelser (6) advocates the weight increase response of the chick thyroid as adaptable to quantitative assays.

The range of structural deviations which we encountered in the guinea pig and chick are exemplified in the accompanying photomicrographs of typical slides used as standards of reference (fig. 1–8). Figure 1 is a photomicrograph of a normal guinea pig thyroid illustrating (*a*) that the colloid of the gland is homogeneous and completely fills the acini, (*b*) that the cells are thin and narrow and stain deeply, and (*c*) that there is no evidence of colloid resorption.

The guinea pig unit (G.P.U.). The minimum quantity of extract injected in 6 equal doses over 72 hours required to effect histological changes in the guinea pig thyroid equivalent to those in figure 2 was designated as the guinea pig unit. The epithelium is definitely higher and there is a definite decrease in stainable colloid around the periphery. If these changes continue, more marked departures from normal are evident as seen in figures 3 and 4. This unit is essentially the Junkmann-Schoeller unit, although younger animals (75 to 150 gm.) were used in this series and our results indicate that they are more sensitive and respond more uniformly than older animals.

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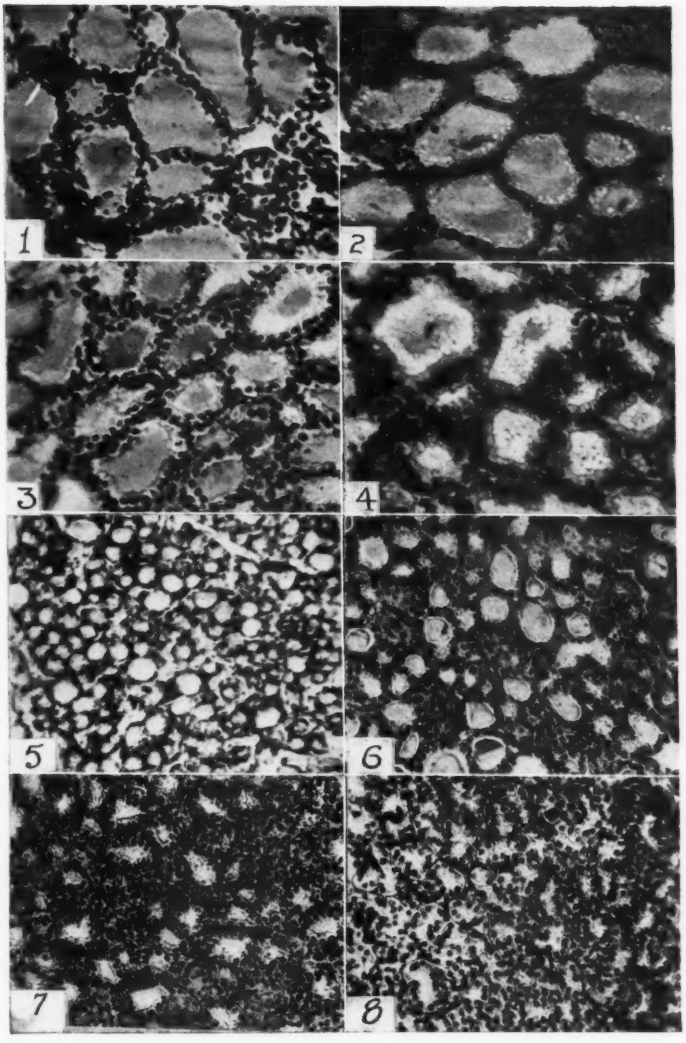

Fig. 1. A NORMAL GUINEA PIG THYROID. Fig. 2. POSITIVE (+) RESPONSE. Definitely active hypertrophy with hyperplasia. Thyroid of a guinea pig illustrating the stimulatory effects of the thyrotropic hormone. Fig. 3. DOUBLE POSITIVE (++) RESPONSE. Moderately active hyperplasia. Guinea pig thyroid illustrating the active resorption and disappearance of colloid and the change in the epithelial cells from a low cuboidal to a low columnar type in sequence of stimulation by the injection of anterior pituitary extracts. Fig. 4. TRIPLE POSITIVE (+++) RESPONSE. Active hypertrophy and hyperplasia. Guinea pig thyroid illustrating the irregular size and distortion of alveoli, the marked liquefaction, the very active absorption of colloid and the high columnar epithelium. Fig. 5. A NORMAL CHICK THYROID. Fig. 6. POSITIVE (+) RESPONSE. Definite hyperplasia. The thyroid of a chick illustrating the effects of the thyrotropic hormone. From the histological standpoint, actual changes in the cells of the chick thyroid are the first indication of stimulation. There is some resorption of colloid but these changes are not conspicuous. Fig. 7. DOUBLE POSITIVE (++) RESPONSE. Moderately active hypertrophy and hyperplasia. Thyroid of the chick, illustrating the change in cell type from the low cuboidal to a columnar type and a marked resorption of colloid. Fig. 8. TRIPLE POSITIVE (+++) RESPONSE. Active hyperplasia. Thyroid of a chick illustrating a complete resorption of colloid in extreme cases, and a complete change of the epithelium to the columnar type.

The chick unit (ch.u.). The chick unit of this study is defined as the minimum quantity of extract injected in 4 equal doses over 48 hours, necessary to effect histological changes equivalent to those found in figure 6. Figure 5 is a photomicrograph of a normal chick thyroid. The acini are very small. The epithelium is very thin and the colloid mass shows no evidences of resorption. A study of figures 2 and 6 indicates that, in contra-distinction to the pituitary effects on the guinea pig thyroid, the first conspicuous indication of stimulation of the chick thyroid is an actual change in the epithelial cells of the acini. If these changes continue, more marked departures from normal are evident as seen in figures 7 and 8.

In our hands, changes in weight of the chick thyroid were considered significant only on the higher levels of stimulation. The nature of the developmental changes in the thyroid indicative of stimulation discouraged the use of borderline dosages.

The preparation of extracts and results. Carefully dissected beef anterior lobes (fresh, frozen or acetone desiccated) and whole sheep pituitary glands were used as a

TABLE 1. COMPARATIVE YIELD OF THYROTROPIC HORMONE FROM PITUITARY TISSUE

Glands used[1]	No. of preps	Assayed on chick[2]	No. of preps	Assayed on guinea pig[2]
		ch.u.		g.p.u.
Undried beef anterior lobes	11	35,100	7	20,680
Undried whole sheep glands	1	5,700	4	6,900
Desiccated sheep glands	1	21,770	1	28,325
Desiccated beef anterior lobes	1	88,200	3	53,300

[1] Glands extracted with $Ba(OH)_2$.
[2] Yield per kg.

source of the thyrotropic hormone. The comparative yield of the hormone from pituitary tissue extracted with 0.02 N $Ba(OH)_2$ appear in table 1. Fresh or frozen sheep pituitary tissue is a poor source of the hormone (6.9 g.p.u. per gm.) whereas good yields can be obtained from beef pituitaries (20.7 g.p.u.). Extraction of the fresh or frozen beef anterior lobes results in the highest yield of thyrotropic activity. The yield from desiccated tissues is approximately 50% of that obtained from tissue when each is compared on the same basis. The yield of undried anterior lobes, for example, from 11.3 kg. of beef pituitaries was 4.4 kg. or 39% of the initial weight of the gland. Following acetone desiccation, the percentage of solids in the final product was found to represent only 8.3% of the initial weight of the gland.

Thyrotropic activity can be obtained in apparently undiminished quantity from either undried sheep or beef anterior lobes which have been stored at $-4°$C. for as long as 7½ months.

Extracts prepared from the ovary of the hog, sheep and beef as well as dosages up to 6,000 r.u. of highly purified preparations of the anterior pituitary-like hormone from pregnancy urine (7) were found to show no thyrotropic activity.

A number of methods of extraction were tried to determine the reagent most suitable for the quantitative extraction since thyrotropic activity can be detected in any simple extract using water (1), dilute NaOH (3), acetic acid buffer mixtures or aqueous solutions containing pyridine, glycerin or urea. Extraction of the hormone using a final concentration of 0.02 N $Ba(OH)_2$ (instead of 0.04 N), a modification of the method of extraction of the growth hormone by Evans, Cornish and Simpson (8) was found to be the most satisfactory reagent for effective removal of the factor from the gland tissue. It does not remove appreciable quantities of the lactogenic hormone. Its use does not cause marked swelling of the tissue. The extracts are quickly and satisfactorily centrifuged, and the barium ion is easily removed.

Attempts to remove inert material contaminating these crude extracts without loss of activity and to increase their potency were made. Experiments utilizing (*a*) differential precipitation with protein precipitants, 2% trichloroacetic acid according to Loeser (2), sulfosalicylic acid (2–3%), picric acid, tungstic, phosphotungstic, flavianic, tannic, picrolonic acids and Reinecke salts; (*b*) salting out procedures; (*c*) alcohol, alcohol-ether or acetone fractionation and (*d*) procedures based on selective adsorption of the hormone were conducted.

It was observed that whenever, in the course of fractionation, recourse to precipitation is made, the precipitate, particularly if it is bulky and flocculent in nature will carry down a high percentage of the active substance from the solution. In the presence of considerable inactive protein this was true of isoelectric precipitation at pH 4.5. The use of flavianic, picric, phosphotungstic, trichloro-acetic and sulfosalicylic

TABLE 2. RECOVERIES OF THE THYROTROPIC HORMONE FROM CRUDE EXTRACTS BY ADSORPTION ON PERMUTIT

Prep. No.	Grams permutit per 100 ch.u. expected	Present	Recovered by elution	Percentage efficiency
		ch.u.	ch.u.	
Pg 69G	20	17,200	<20,858 >15,090	Approaching 100
Pg 69A	24	2,760	2,534	92
Pg 68A	20	17,920	14,520	80
Pg 66D	17	12,821	11,584	90
Pg 70	20	20,250	18,000	90
Pg 64A	22	15,556	16,900	100
Pg 66C	12	1,550	1,320	85
Pg 66A	16	621	572	92
Pg 66B	8	621	440	70

acids on crude extracts resulted in removing 60 to 90% of the active factor together with most of the contaminating protein. In more extensively purified preparations, these reagents yielded little or no precipitate and the supernatant solutions contained nearly all of the original activity. Evans and coworkers reported that the thyrotropic principle was precipitated by flavianic acid but Smelser (9) found the preparation to contain "a great deal of gonadotropic, adrenotropic, and growth hormone activity." In our opinion, flavianic is a much more satisfactory precipitant for the growth hormone. Its use did not offer a satisfactory step in the separation or purification of the thyrotropic hormone from the other pituitary factors and provided the additional difficulty of effective removal of the flavianic acid from the preparation.

Our every attempt at clarification or purification of the crude extracts confirm the conclusion of Junkmann and Schoeller that the hormone is carried down by colloidal precipitates which make satisfactory recoveries difficult. The ease with which the thyrotropic hormone was adsorbed led us to perform experiments to determine the usefulness of various adsorbents, namely: hyflo, kieselguhr, alumina gel, kaolin Lloyd's reagent, benzoic acid (7), norite, permutit and several adsorbents of a similar composition. We found the hormone to be adsorbed on a variety of these adsorbents. Poor recoveries followed the use of charcoal, celite and Fuller's earth due apparently to difficulty in effecting an adequate elution of the hormone from the adsorbent. The adsorbents, Zeo-Dur, Decalco 30/50, Decalco 20/10, and Super Zeo-Dur in contradistinction to permutit failed to remove the hormone satisfactorily from solution. The use of permutit was considered to represent progress in the choice of methods of purification. It was observed that 15 to 20 gm. of permutit per 1,000 ch.u. of thyrotropic activity was required for the quantitative removal of the hormone. Optimal

adsorption occurred at pH 4.5. The permutit settled out rapidly allowing the supernatant solution to be decanted. It was easily washed free from contaminating extract and isoelectrically precipitated protein using water acidified with acetic acid, and the activity could be eluted satisfactorily by alkali.

Some results of experiments conducted on crude extracts using permutit appear in table 2. The permutit was added to the extract, the pH adjusted to 4.5 and the solution vigorously stirred for one hour with a mechanical stirrer. The permutit which

TABLE 3. EFFICIENCY OF A PERMUTIT ADSORPTION CONDUCTED ON PARTIALLY PURIFIED PREPARATIONS

Prep. No.	Grams permutit per 1,000 ch.u. expected	Present	Recovered by elution	Efficiency
				%
Pg 58C	28	12,434 ch.u.	10,960 ch.u.	88
Pg 60	21	16,226 ch.u.	>10,520 ch.u. <12,400 ch.u.	>65
Pg 64A_3	20	2,900 ch.u.	2,478 ch.u.	80
Ps 51C8	40	833 g.p.u.	729 ch.u.	88 (?)
Pg 66D_3	35	2,000 ch.u.	2,116 ch.u.	100
Ps 52C_1	30	1,350 g.p.u.	1,350 ch.u.	100 (?)
Pg 88B	17	>11,145 g.p.u. >11,454 ch.u. <17,045 ch.u.	12,867 g.p.u. >7,720 ch.u.	>70
Pg 86C	17	22,845 ch.u.	26,718 ch.u.	100
Pg 85C	17	<29,350 ch.u.	23,650 ch.u.	80

ch.u., Chick units
g.p.u., Guinea pig units
Adsorptions conducted at pH 4.5

selectively adsorbs the thyrotropic hormone settles out instantly allowing it to be separated and washed by decantation. An additional quantity of the hormone was recovered from the mother extract. Acidification of the crude extracts caused the isoelectric precipitation of protein. To obviate the loss of activity adsorbed on this precipitate, we found it advisable to redissolve the protein in the mother extract, by the addition of 2 N alkali and repeat the permutit adsorption.

The rather large volume of permutit required to effect adequate removal of the hormone required the use of a considerable volume of alkali for adequate elution. Furthermore, we observed that the pH must be carefully controlled during the period of elution; otherwise the hormone was not eluted. We found that the activity could be satisfactorily recovered by the dropwise addition of 2 to 2.5 N NaOH to pH 13 followed by 3 or 4 extractions with 0.05 N NaOH. Such preparations contained 10 to 20 ch.u. of the thyrotropic hormone but also contained the growth factor in appreciable quantity.

These preparations were further purified by merely repeating the permutit adsorption. The percentage recovery as shown in table 3 is very good since 80 to 100% of the thyrotropic activity was recovered from the adsorbent. Furthermore, no growth or gonadotropic activity could be detected in these preparations. Further purification procedures based on adsorption of the hormone on permutit were found to be impracticable. It would appear that the presence of impurities is essential for the adsorption of the hormone on permutit since recoveries were poor.

Precipitation of the thyrotropic factor with uranium acetate proved the most satisfactory of additional attempts to effect further concentration and purification of these extracts (table 4). Uranium acetate was added to the product of the third permutit adsorption to give a final concentration of 0.1 to 0.12%, the pH adjusted to 8.5

TABLE 4. URANIUM ACETATE PRECIPITATION

Prep. No.	pH	Units present	Filtrate recovery	Recovery from precipitate	Recovery in precipitate
					%
Pg 75	5.0	10,000 ch.u.	—	5,850 ch.u.	58
Pg 66C2	6.0	1,300 ch.u.	—[1]	1,252 ch.u.	88
Pg 58C5	7.2	2,680 ch.u.	290 ch.u.	>2,400 ch.u.	>89
Pg 82A	8.5	15,210 ch.u.	—	7,120 ch.u.	50
Pg 85C	8.5	23,650 ch.u.	—[1]	>18,575 G.P.U. >14,925 ch.u.	>62
Pg 88D	8.5	13,426 G.P.U.	1,000 G.P.U. 800 ch.u.	10,350 G.P.U. <11,638 ch.u.	77
Pg 69Y	8.5	1,200 G.P.U.	—[1]	1,279 ch.u.	100 (?)
Pg 90	8.5	>7,950 ch.u.	—	8,766 ch.u.	100
Pg 86C	8.5	>26,250 ch.u.	—	36,250 ch.u. >13,850 G.P.U.	100
Pg 64	8.5	11,400 ch.u.	—	12,000 ch.u.	100
Pg 96	8.5	32,960 ch.u.	<460 ch.u.	<2,834 ch.u.	<8[2]
Pg 98	8.5	5,000 ch.u.	>660 G.P.U. 240 ch.u.	>1,400 G.P.U. <1,800 G.P.U. >1,260 ch.u.	28[2]

[1] Filtrate too dilute to demonstrate presence of activity.
[2] Uranium precipitation conducted a second time. There is always a marked loss of activity.

and the precipitate removed by centrifugation. The hormone was most satisfactorily recovered and the uranium removed from this precipitate by the aid of three or four extractions of 3% NaH_2PO_4. Extracts obtained by this method always contained 100 or more G.P.U. or ch.u. per cc. A repetition of the uranium acetate precipitation of the hormone as a method of purification was not satisfactory (Pg 96, 98, table 4).

SUMMARY

Data have been presented to indicate that sheep pituitaries are a poor source of the thyrotropic factor whereas good yields can be obtained from beef pituitaries. A method of preparation for potent extracts of the thyrotropic hormone has been presented. This consists of the extraction of the hormone with 0.02 N $Ba(OH)_2$, the differential adsorption of the hormone using permutit at pH 4.5 and its subsequent concentration by precipitation with uranium acetate. The hormone is characterized by the fact that it is adsorbed on a variety of substances.

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THYROID HORMONE IN BLOOD AND TISSUES IN RELATION TO BASAL METABOLIC RATE[1]

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THE STUDY of thyroid hormone was begun 10 years ago (1) and in collaboration with J. W. Cavett it was observed that thyroglobulin was purified by repeated solution and reprecipitation (2). The results of this paper together with the second of the series (3) tended to show that thyroxin is built up within the thyroglobulin molecule, since the number of molecules of tyrosine+diiodotyrosine+thyroxin was constant within experimental error while the thyroxin content was increasing from 0.005 to 0.35%. (Schmidt, (4), expressed the same view of the formation of other proteins.) The senior author took these thyroglobulins to Svedberg's laboratory and found them to be polydisperse in comparison with thyroglobulin forced out of living thyroid glands by centrifugal force(5). Hektoen, Carlson and Schulhof by precipitation reaction found thyroglobulin in the blood of the thyroid vein but not in other locations in dogs not fed thyroid (6).

EXPERIMENTAL

Since the thyroid hormone does not seem to be active physiologically while it remains in the thyroid gland it would be interesting to determine its concentration in the blood and other tissues. Although several investigators have attempted to do this (7) our own seems to be the only work in which the results are correlated with the basal metabolic rate, which is the standard test of thyroid function. We must admit that there are error in B.M.R. determinations and in our thyroid hormone determinations but it seems best to show the correlation before discussing details of sources, of error. If the B.M.R. is denoted in calories per square meter per hour (to eliminate age and sex) and the thyroid hormone iodine recorded as micrograms per 100 cc. of blood, 373 cases are distributed as shown in table 1.

Thus between 27 and 30 calories per sq. m. per hr. there were 5 patients with between 1 and 2γ and 2 patients between 2 and 3γ of thyroid hormone iodine per 100 cc. blood. The mean[2] of the calories per sq. m. per hr. was 41.84 ± 0.36. The mean of the micrograms was 5.2 ± 0.08. The correlation coefficient was $+0.627$ which is fairly high considering the fact that it is between two rather distantly related quantities on highly complex organisms.

Another test was made by feeding desiccated thyroid to *J. F. M.* whose B.M.R. had repeatedly been determined as shown in table 2.

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[1] Aided by a grant from the Elizabeth Thompson Science Fund. Thanks are due to Drs. Gustave van Lennep and G. Harlan Wells and their staffs and Hahnemann Hospital for blood specimens and basal metabolic rate determinations.
[2] Forty cases were added after calculations were made by method of Pearl, Introduction to Medical Biometry and Statistics, pp. 366–393, 2nd Ed., Philadelphia (1930).

TABLE 1

γ per 100 cc.												
12	□	0	□	0	□	0	0	0	3	2	1
11	□	0	0	0	0	□	0	□	3	3	1
10	0	0	0	0	□	1	1	0	2	3	2
9	0	0	0	□	2	4	2	6	0	4	□
8	0	0	□	5	9	4	3	4	3	4	3
7	□	0	4	10	9	5	4	6	2	2	5
6	0	4	5	12	13	10	7	2	3	3	3
5	0	2	14	11	9	11	4	3	1	4	0
4	0	7	10	18	10	7	9	2	0	□	0
3	2	3	20	14	4	3	2	□	0	0	□
2	5	3	6	5	0	0	□	□	0	0	0
1	27	30	33	36	39	42	45	48	51	54	57	60

Calories per square meter per hour.

It is thus seen that (although the thyroid hormone in the blood rises higher during the first day after taking 50 grains of desiccated thyroid than the B.M.R. rises) after 5 days the B.M.R. rose 25% in the 1st exp. and 28% in the second whereas the thyroid hormone rose 30% in the first and 35% in the second, or in as good agreement as one could expect in such experiments.

In another experiment W. C. F. took 2.5 gm. of potassium iodide, a dose which caused his salivary glands to swell. His thyroid hormone iodine was 5γ/100 cc. blood before taking the dose and 5γ/100 cc. three days after taking the dose.

A sample of beef blood was divided into 2 parts and to one part was added 1000γ of I^- per 100 cc., then both parts were analyzed and found to contain 4γ of thyroid hormone iodine per 100 cc.

TABLE 2

Preliminary study	Cal./sq.m./hr.	Thyroid hormone iodine in 100 cc. blood	Dose of desiccated thyroid taken after B.M.R. determination
Aug. 6	37.5		
27	38.5		
28	33.2		
29	32.2		
Nov. 15	36.1		
1st. exp.			
Mar. 15	34.5	4.0	50 grains
16	42.2(+22%)	6.0(+50%)	
20	43.3(+25%)	5.2(+30%)	
2nd exp.			
June 7	32.2	4.0	50 grains
12	41.0(+28%)	5.4(+35%)	

In another study a patient was prescribed 1 gm. diiodotyrosine per day by her physician. An analysis of the blood showed no rise in the thyroid hormone.

To demonstrate the recovery of thyroxin iodine by this method, 10,000γ of thyroxin containing 6540γ of iodine was dissolved in a liter of dilute NaOH, and 1 cc. of the solution added to 99 cc. defibrinated beef blood (sample 1). To another 99 cc. of the same blood was added 1 cc. water (sample 2). The analytical results were as follows.*

TABLE 3

	γ iodine in 100 cc.
Sample 1 (blood+6.54γ iodine added as thyroxin)	10.12
Sample 2 (blood)	4.44
Thyroxin iodine recovered	6.68

In another experiment 209.1 mg. thyroglobulin containing 0.287% iodine (600γ) was dissolved in 100 cc. water and 1 cc. added to each of two 100 cc. flasks and 1 cc. water to each of 3 others.

TABLE 4

	γ/100 cc.	Average
Sample 1 (+6γ thyroglobulin iodine)	11.12	
Sample 2 (+6γ thyroglobulin iodine)	10.20	10.66
Sample 3 (no thyroglobulin added)	4.44	
Sample 4 (no thyroglobulin added)	4.40	
Sample 5 (no thyroglobulin added)	4.40	4.41
	Difference	6.25

The 4% error in analyzing the thyroid hormone is as small as can be expected.

Although potassium iodide did not interfere with the determinations of thyroid hormone, very abnormal results were obtained on patients treated with lipiodol or mercurous iodide. Since mercurous iodide is not removed by our technic and diiodotyrosine is, it does not seem more accurate to call the results "organic iodine." With the precaution of excluding patients treated with certain iodine preparations, the evidence so far indicates that we are analyzing thyroid hormone iodine.

Since the treatment of casein with elemental iodine may result in the formation of thyroxin (8) we have tried to exclude blood from patients treated within 2 weeks with Lugol's solution or other elemental iodine preparations, and we suggest the substitution of 15% potassium iodide solution for patients who may later be studied by this technic.

The above evidence that we are analyzing thyroid hormone outside the thyroid glands is considered justification for the description of our technic, as follows.

METHOD

Samples of blood or other tissue large enough for duplicate analyses are obtained in the fresh state. Eleven to twelve cc. of blood are collected in a tube containing 0.07 to 0.1 cc. of 30% potassium oxalate and inverted to mix. Five cc. is drawn up in a fine pointed pipet and with the point just beneath the surface of 100 cc. methanol in a 125 cc. glass stoppered Erlenmeyer flask, it is blown out in the form of a fine spray. (In case any visible lumps appear it is homogenized 5 minutes in a Waring blendor.)

Tissue is weighed and 5 gm. samples are placed in 1 oz. bottles and then placed

* Blood stood a week or more before analysis and thyroxin may have combined with protein. When analyzed immediately recovery is not complete.

in the freezing unit of an electric refrigerator for freezing and storage. When a sufficient number have accumulated the samples are placed one at a time in a steel mortar which is then filled with liquid nitrogen. A plunger is inserted as quickly as possible and when the liquid nitrogen has boiled away, as evidenced by the cessation of the escape of gas, the plunger is struck with a 6 lb. sledge hammer.

A convenient mortar is made by boring a hole in one end of a 6″ piece of fence post to receive a 2″ cap to a water pipe. The post is then imbedded in a 16″ or 18″ cube of concrete that has been formed in a box. The box in which a carboy has been packed is convenient. If casters are fitted to the bottom of the box it may be transported more easily. The plunger may be made of a short piece of 2″ steel shafting.

Immediately after striking with the sledge hammer the tissue is placed in 100 cc. of chilled menthanol in a Waring blendor and homogenized 5 minutes. (If liquid nitrogen is not available, frozen tissue may be homogenized in ice cold methanol in a Waring blendor but at least 10 minutes are required and during that time heat is generated and some cooling mechanism is desirable.) A stopper inserted into the blendor and coming to within 1 cm. of the top of the methanol is an advantage, but since the blendor is clover-leaf shaped in cross section it is difficult to obtain a close fit with a stopper. (The jar of the blendor might be cut off at an internal height of 5 cm.) After homogenizing, the sample is placed in a 125 cc. glass stoppered Erlenmeyer flask.

The ~~flasks are placed in a shaking or rotating machine for 1 hour, after which the~~ tissue is allowed to settle and the methanol decanted.

After at least 85% of the methanol has been removed 100 cc. of acetone is added and the flask agitated and the contents poured into a 100 cc. buret shaped tube, 1 meter long, 12 mm. bore with the lower end narrowed to 7 mm. To this end is attached 9 inches of 3/8″ Visking sausage casing.[3] The lower end of the casing is plugged with 1″ of grapevine. The acetone does not filter through the sausage casing at an appreciable rate, but drips through the vascular bundles in the grapevine.

When the acetone has disappeared and the precipitate is in the casing, the casing is allowed to dry in air and then tied at both ends and the grapevine and buret cut off. The casing is flattened between 2 sheets of clean paper and a knitting needle pressed lengthwise into one side. It is wound with sewing cotton and the needle withdrawn. The casing is dried in an oven at 140° C. for 2 hours. The sample is then analyzed for iodine by the McClendon-Bratton method (9).

A few modifications have been made in this method.[4] A Telechron motor (B-2 1 R.P.M. 60 C) is easily attached to the screw feed with a rubber tube. We do not supply CO_2 to the stoking tube, but use nearly 200 cc. of oxygen per minute.

After distillation, 10 minutes of rapid aeration at 100° are required to blow off the excess bromine. The original directions called for 5 minutes after the color of bromine disappeared, but this latter is hard to determine. The aeration must be so rapid that the solution is evaporated to about 2 cc. If more than 3 cc. remain, the aeration should be prolonged. A slightly less amount of aeration may cause a large error. One must observe that the aeration does not stop during the 10 minutes and if a number of tubes are aerated from one air supply, that aeration is equal in all tubes.[5]

[3] From Fisher Scientific Co., Pittsburgh.

[4] The platinum parts and stainless steel stoking tube may be obtained from Bishop & Co., Malvern, Pa., the microburet from Williams, Brown and Earle, Philadelphia, the absorber and the flow-meter from Corning Glass Works, Corning, N. Y.

[5] A capillary manifold for aerating 4 tubes from one air supply may be obtained from the Precision Thermometer and Instrument Co., 1434 Brandywine St., Philadelphia, who also make an improved form of microstill.

If aeration is excessive in any tube it may be reduced by adding iodine-free water to that tube.

RESULTS

We have had to eliminate certain data on patients where duplicate analyses of the thyroid hormone in the blood or duplicate determinations of the B.M.R. were very divergent. After these eliminations there remained 373 cases which have been tabulated (table 1). There are 3 possibilities to explain the considerable spread to the data: there may be actually no higher correlation between thyroid hormone in the blood and B.M.R. than shown by this data; there may be errors in the determination of the thyroid hormone and there may be errors in the determination of the B.M.R. It is this last possibility that makes the determination of the thyroid hormone in the blood useful in diagnosis.

Since the metabolism of the blood itself is very low, the significance of the thyroid hormone in it is that it is being transported to the other tissues. It would be more useful to know the thyroid hormone of muscle, liver, heart and other tissues that make up a large percentage of the volume of the body. But we do not recommend biopsy for this purpose. The data we have are entirely on autopsy and the basal metabolic rates were not known. In 2 cadavers blood showed 57γ/100 cc. and the average of the tissues was 4.9 and 4.6γ/100 gm. These tissue values would be about 5.2 and 4.9γ/100 cc. if corrected for density (about 1.06).

The main interest is in the average figures. We have not been able to entirely eliminate errors due to iodine containing drugs. Often the medical record can be traced back only a few weeks. We believe that mercurous iodide, for instance, may concentrate in the liver and kidney and may remain for long periods. Since young individuals have not had as much time for such accumulations as older ones have had, we divide the data according to age (under or over 30 years of age).

In 6 autopsies of those under 30 years of age and 25 autopsies of those over 30 the following average figures were obtained.

TABLE 5. MICROGRAMS ~~THYROXIN~~ thyroid IODINE/100 GM.

Age, years	Muscle	Heart	Liver	Kidney
<30	6.2	6.4	5.8	5.8
>30	5.1	5.9	7.7	6.6

It thus appears that although the thyroid hormone in the muscles and heart decreases with age as does the B.M.R., the results with the liver and kidney appear to increase with age. We believe that this appearance is due to more numerous positive errors in the higher age group due to accumulation of such drugs as iodide of mercury in the liver and kidney.

Whereas beef muscle had the same thyroid hormone iodine as the younger human being (6.2), smaller animals with higher B.M.R.'s per unit weight had higher values. Thus the values for the mouse were 24 in muscle and 17.2 in liver and for the rat were 9.4 in muscle and 8 in liver. A rat fed 20 grains of thyroid (which it took 2 days to eat) had 10.4 in the muscle and 58 in the liver.

Pathologists have found teratomata in the ovary which had the appearance of thyroid tissue and consequently gave the name struma ovarii. We obtained 2 specimens and found them to average 486γ thyroid hormone iodine per 100 gm, or about 97 times the average value for the blood. Another piece of thyroid-like tissue which was considered to be the metastasis of one of the above struma ovarii in the ischium contained 323γ thyroid hormone iodine per 100 gm.

It has been thought that the hypophysis contains thyroid-like colloid and some work has been done on its iodine content (10). Baumann and Metzger (11) found it to contain no more iodine than other tissues (excepting the thyroid). Owing to its small size we did not attempt to fractionate the iodine of the hypophysis but have determined the total iodine as follows.

TABLE 6

Age	Sex	γ total iodine/100 gm.
55	M	10
35	M	20
21	F	23
50	M	29
7	M	40
54	F	54
18	F	56
3	F	75
50	M	90
	Average	44

The average is about 1% of that found in the thyroid and in agreement with Baumann and Metzger on a par with that found in other organs (1).

DISCUSSION

The blood iodine fractionation method described seems to bear a relation to thyroid function. The term, thyroid hormone iodine, or for brevity, thyroxin iodine, seems appropriate. On the other hand, the term, organic iodine, does not seem suitable since free diiodotyrosine is not included in this iodine fraction and mercurous iodide (if present) is.

There are sources of error in determining both thyroxin iodine and basal metabolic rate. The most serious error in the thyroxin determinations is failure to blow out all the residual bromine, giving + errors. The most common error in B.M.R. determinations is increased muscular tone of the patient (due to apprehension) and is likewise a + error. Even a calm person will show a progressive decrease in the B.M.R. for a week if it is taken every day. Blood thyroxin iodine values of more than 12γ per 100 cc. should be thrown out unless they can be confirmed with repeated blood samples and check in duplicate. B.M.R. determinations above +50 should be ruled out unless they can be repeated every day for a week.

The tissue thyroxin determinations are confirmation of the blood findings.

Owing to the meticulous care needed in our iodine analyses it is interesting to note that Trevorrow and Fashena (12) and Riggs and Man (13) have attempted to develop easier methods. It is our belief, however, that no method should be judged by the total iodine of blood determined by it. We have never been able to establish any constancy in total blood iodine such as is true of its osmotic pressure, chloride content, alkali reserve or pH. We have found blood to contain as high as 3,700γ of total iodine per 100 cc. for weeks. This was largely due to 15 gm. sodium iodide per day absorbed from the intestine. Whereas on continuous intravenous injection of $NaCO_3$ the pH of arterial blood was determined by the senior author to be normal, and continuous injection of HCl did not reduce the alkali reserve more than a small fraction of the calculated amount, there seems to be no regulating mechanism for total blood iodine.

SUMMARY

With the exception of patients treated with certain iodine drugs other than potassium iodide and diiodotyrosine we are able to extract from the blood all forms of iodine except thyroid hormone iodine. The latter may be called thyroxin iodine for brevity, with the proviso that we leave open the questions of the structure of thyroglobulin and the assay of thyroid preparations. Outside the thyroid it seems probable that thyroxin is more or less equally distributed on the average.

From a study of the blood and basal metabolic rates of 373 patients and the study of tissues from numerous autopsies we have found that for every microgram of thyroxin iodine in 100 cc. blood there is about 1 mg. of thyroxin in the body of a 65 kg. adult and the metabolic rate in percentage of normal, averages roughly as follows.

TABLE 7

B.M.R.	−30	−20	−10	0	+10	+20	+30	+40	+50
γ thyroxin iodine in 100 cc. blood	2	3	4	5	6	7	8	9	10
Mg. thyroxin in body of 65 kg. adult	2	3	4	5	6	7	8	9	10

Therefore the determination of thyroxin iodine in the blood is a check on the reliability of the B.M.R. determination and is a substitute in case the B.M.R. cannot be determined. The use of elemental iodine (as in Lugol's solution), iodide of mercury or lipiodol leads to error.

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THE EFFECT OF ADRENALECTOMY ON PREGNANCY AND SURVIVAL OF UNTREATED AND SESAME OIL TREATED RATS

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NORMAL PREGNANCY has been reported as not possible for the adrenalectomized rat (1, 2). Wyman (3) and Gaunt (4), however, found normal pregnancy could occur in a small percentage of adrenalectomized rats in the absence of gross rests of cortical tissue, while others (5, 6) reported no change in the course of pregnancy of their adrenalectomized animals.

The cases in which normal gestation and parturition have been found in the adrenalectomized rat, in the absence of macroscopic rests of cortical tissue, have been attributed to the maintenance of the animal during the course of pregnancy by cortical hormone either secreted by microscopic rests of cortical tissue or residual in the animal's tissues after adrenalectomy. Recent work, however, has firmly established the beneficial effect of pregnancy and the hormone of the corpus luteum in the adrenalectomized animal. Pregnancy and pseudopregnancy, natural or induced, have been found to prolong the survival time after adrenalectomy in several species (2, 7–12). Excessive luteinization of the ovaries and substitution of other hormones for the hormone of the adrenal cortex, particularly progesterone, have also been shown to increase the survival time of adrenalectomized rats (13–19).

McKeown and Spurrell (2) have shown that the survival time of pregnant rats from which the adrenals and fetuses were removed, but the placentas left intact, was longer than that of non-pregnant adrenalectomized animals. Others (20–22) have also found that the placento-fetal complex in pregnant rats with reduced number of fetuses will maintain pregnancy, indicating that this complex is a possible source of progesterone-like substances which might therefore substitute for the adrenal cortical hormone in the adrenalectomized pregnant rat.

In view of these findings, the following experiments were performed. (*a*) Rats were adrenalectomized before mating or on the second or 17–19th day of pregnancy to determine whether the hormones of pregnancy would permit normal gestation in the rat in the absence of tissue of the adrenal cortex. (*b*) Female rats were sub-totally adrenalectomized to test whether residual cortical tissue enables the rat to have normal reproduction and to survive indefinitely. (*c*) The possibility that the placento-fetal complex is a source of hormones which may substitute for the adrenal cortical hormone and maintain the adrenalectomized rat during pregnancy was tested by adrenalectomizing and ovariectomizing a group of pregnant rats.

It has been found (Tobin, unpublished data) that daily injections of sesame oil benefit pregnant adrenalectomized rats. The effect of injections of sesame oil was tried, therefore, to ascertain whether this oil would affect normal reproduction or the course of survival of rats adrenalectomized before or during pregnancy.

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Pregnancy and Survival of Untreated and Sesame Oil Treated Adrenalectomized Rats

Female rats were adrenalectomized before mating or on the second or 17–19th day of pregnancy (table 1).[1] Pregnancy was dated from the morning on which the vagina was found to contain a copulation plug or spermatozoa and was later verified by the presence of implantation sites in the uterus. The animals not having implantation sites at autopsy were discarded. This precaution avoided using animals which had sterile matings.

The death of the females with typical symptoms of adrenal insufficiency was the criterion of complete adrenalectomy. Only those animals which ultimately died of adrenal insufficiency were used in this experiment, although the other animals which lived indefinitely and were found to have residual cortical tissue at autopsy are also tabulated in table 1 B for the purpose of a subsequent discussion.

TABLE 1. PREGNANCY IN ADRENALECTOMIZED RATS

Time of operation	A Complete adrenalectomy			B Residual cortical tissue		
	No. animals	No. pregnancies	No. litters[1]	No. animals	No. litters	
					Delivered	Weaned
Day before exposure to males	38	14	3	5	4	2
2nd day of pregnancy	40	40	16	7	7	5
17–19th day of pregnancy	25	25	23	10	10	4

[1] Litters normal at birth died before weaning.

Adrenalectomized before mating. Forty-three females were adrenalectomized and were placed in cages with normal males the day following operation. Five of these females lived indefinitely and were found to have residual cortical tissue at autopsy. The other 38 females died of adrenal insufficiency (table 1). Seventeen of the 38 females were given intraperitoneal injections of 0.25 cc. sesame oil daily until the time of death or, in the case of those which had successful gestation, until the time of delivery. The other 21 animals were not given any treatment. Vaginal smears, which were made from these females to the time of impregnation or death, showed that their estrous cycles were irregular after the first week. Most of the animals which became pregnant were inseminated within the first week after operation. In only 2 cases did insemination occur later, both on the 15th day after adrenalectomy. Toward the latter part of the survival period all the non-pregnant animals showed continuous diestrous smears.

Twenty-four of the animals (15 controls and 9 oil treated) did not become pregnant. The other 14 females (6 controls and 8 oil treated) were impregnated (table 2 A), but only 1 control and 2 oil treated animals delivered. The other 11 females resorbed their young within the first half of the gestation period.

The survival time of the animals which did not become pregnant in the oil treated group did not differ significantly from that of the control group; nor did the survival time of either of these 2 groups differ from that of adrenalectomized, mature non-pregnant females of this colony, which is 12.5 ± 2.3 days. The average survival of the pregnant females treated with oil, whether or not they resorbed their young, was longer than that of the pregnant controls (table 2 A). The litters from the 2 groups did

[1] All the animals used in these experiments were kept in a thermostatically controlled room (80°F.) and given tap water and the standard diet for this colony, Purina Fox Chow, ad libitum.

not have significantly different survival times, since they all died within the first week after birth.

Adrenalectomized on the second day of pregnancy. Forty-seven females were adrenalectomized on the day after finding a copulation plug or spermatozoa in the vagina. Seven of the animals lived indefinitely and were found to have residual cortical tissue at autopsy; the other 40 animals died of adrenal insufficiency (table 1). These 40 animals were in 2 groups of 20 each: one group received no treatment and the other group received daily injections of 0.50 cc. sesame oil intraperitoneally until the time of death or delivery.

TABLE 2. SURVIVAL OF FEMALES AND LITTERS

	Uninjected				Sesame oil 0.25 cc. daily				Mean Diff.
	No. animals	Mean	P.E. days	σ	No. animals	Mean	P.E. days	σ	P.E. Mean Diff.
A. Adrenalectomized day before exposure to males									
Non-pregnant females	15	11.5	±0.9	±5.3	9	13.8	±1.2	± 5.3	1.5
Pregnant females	6[1]	18.6	±2.4	±8.8	8[2]	27.4	±3.0	±12.7	2.3
Litters	1	5.0			2	7.0			
B. Adrenalectomized second day of pregnancy									
					0.5 cc. daily				
Females (delivered)	7	36.3	±1.7	±6.8	9	53.8	±3.7	±16.6	4.2
Litters	7	3.2	±0.6	±2.3	9	8.8	±1.8	± 8.1	3.3
Females (resorbed young)	13	18.1	±1.4	±7.7	11	14.5	±0.9	± 4.9	2.2
C. Adrenalectomized 17–19th day of pregnancy									
					0.5 cc. daily				
Females	10	13.2	±0.7	±3.2	15	24.2	±1.7	± 9.5	6.1
Litters	10	5.5	±0.3	±1.6	13	7.6	±1.0	± 5.4	2.0

[1] 1 pregnant female (delivered) survived 35 days
[2] 2 pregnant females (delivered) survived 44 and 49 days

$$\sigma = \sqrt{\frac{\Sigma d^2}{n-1}}$$

Sixteen of the females (7 control and 9 oil treated) delivered normal litters, whereas the remaining 24 females (13 control and 11 oil treated) resorbed their young within the first half of the gestation period. The number of successful pregnancies was 10% higher for the oil treated females than for the controls, indicating that the oil injections were beneficial in maintaining normal pregnancy in rats adrenalectomized at this stage of pregnancy (table 2 B).

The survival time of both the oil treated and control groups which delivered shows that successful gestation increases the survival time of rats adrenalectomized at this stage of pregnancy (table 2 B) over the survival time of the groups adrenalectomized before becoming pregnant (table 2 A). In the oil treated group, the females which delivered had an average survival time approximately 4 times as long as the females which resorbed their young; while in the control group, the animals which delivered lived approximately twice as long as those in which resorption occurred.

The average survival time of the oil treated animals which resorbed their young was shorter than that of the controls. This was the only case in which injections of sesame oil did not prolong the average survival time of adrenalectomized pregnant rats, since these oil treated females which resorbed their young lived approximately the same average length of time as the mature non-pregnant females of this colony.

The litters from the oil treated females lived more than twice as long as the control litters. In one case, 3 young of a litter of 7 from an oil treated female survived 22 days after birth. These results suggest that sesame oil residual in the animal also enhances the ability of the adrenalectomized rat to maintain her young after delivery.

Adrenalectomized on the 17–19th day of pregnancy. Thirty-five animals were adrenalectomized during the last quarter of the gestation period. Ten of these females had residual cortical tissue at autopsy and were not included in the results of this experiment. The other 25 animals died of adrenal insufficiency (table 1). Fifteen of these 25 were injected intraperitoneally with an average dose of 0.50 cc. sesame oil daily (table 2 C). These injections were given over a period of 8 to 25 days after adrenalectomy.

Twenty-three (10 control and 13 oil-treated) females gave birth to normal litters. The other 2 females from the oil treated group gave birth to full-term dead young. The failure of these 2 females to give birth to living young is attributed to the mechanical factors involved in the handling and giving of the intraperitoneal injections of oil at this stage of pregnancy when the normal birth process could easily be deranged by such treatment.

The survival time of all the oil treated animals was approximately twice as long as the controls (table 2 C); whereas the survival of the controls was not significantly different from the average survival of the nonpregnant adrenalectomized females of this colony.

The survival time of the litters from the control and oil-treated females adrenalectomized at this stage of pregnancy was not significantly different except in two cases in which some young from oil treated mothers lived more than 2 weeks. One female carried 4 young of a litter of 8 to the 17th day and the other female, 3 young of a litter of 7 to the 19th day after birth. Both of these litters were stunted and approximately one-half the weight of normal young of their age at the time of death. No direct correlation was found between the survival time of the mothers and their young. As compared with the survival time of the control females and their litters, the oil injections increased the survival time of the treated females and in a few cases, enhanced their ability to maintain normal young after delivery.

Effect of Sub-total Adrenalectomy on Survival and Reproduction

The following experiments were tried to see whether residual tissue of the adrenal cortex resulting from incomplete adrenalectomy would maintain life and normal reproduction in the rat. Mature animals were sub-totally adrenalectomized by removing the whole of one and more than half of the remaining adrenal. The unremoved fragment was left in place and care taken not to injure its normal blood supply.

Twenty-two females were incompletely adrenalectomized and isolated from males for a period of 80 days. Vaginal smears made from these animals showed that their estrous cycles were normal in all except 4 cases. These 4 females had periods of diestrus within the first month after operation, which lasted from 11 to 13 days. On the 80th post-operative day, 9 females were killed and the other 13 placed in cages with normal males. All 13 animals became pregnant within one week and subsequently bore normal litters. Although one young was lost from each of 3 of these litters, all the remaining young were carried to weaning.

Six other females were also sub-totally adrenalectomized and placed in cages with normal males the day following operation. Two of these females were inseminated on the 3rd and one on the 17th, 35th, 62nd and 79th day after being placed with males. The litter from the animal inseminated on the 17th day survived 3 days. Two young were lost from each of 2 other litters whereas all the remaining young were normal in weight and development at the time they were weaned.

Twenty-two of the females adrenalectomized before or during pregnancy lived indefinitely. They were found to have residual cortical tissue at autopsy (table 1 B) and therefore were not used in the data of the first experiment. Twenty-one litters were born from these females but only 11 of these litters were carried to weaning.

All the females from these three groups lived indefinitely. These experiments show that residual cortical tissue will maintain the life of the incompletely adrenalectomized rat and, if this tissue is given sufficient time to regenerate, the animal will have normal reproduction. These results are additional evidence that the animals used in the first experiment did not have residual cortical tissue which atrophied and ceased to maintain life after delivery; for all the animals used in that experiment ultimately died of adrenal insufficiency whereas the animals from these groups which had residual cortical tissue lived indefinitely.

Bilateral Ovariectomy and Adrenalectomy during Pregnancy

In order to test whether the placento-fetal complex influenced the pregnancy and survival of the animals used in the first experiment a group of rats had both ovaries and adrenals removed during pregnancy, either by a single-stage operation or by a 2-stage operation with 2 days intervening before the removal of the contralateral ovary and adrenal.

Sixteen animals were used and their ovaries and adrenals were removed at periods ranging from the 9th to the 17th (average 12th) day of pregnancy. The number of fetuses was not reduced nor were any of these animals treated with sesame oil. In only 2 cases was gestation completed. Both of these animals died delivering normal young. There was some fetal growth in all the other animals, ranging from 2 to 8 days before resorption or abortion occurred. The average survival time of 8 animals which had single-stage operations was 11.7 days, while the other 8 animals which had 2-stage operations survived 7.8 days.

It can be seen from this experiment, as compared with the results of the first experiment, that the presence of the fetuses and their placental complex was not sufficient to prolong survival or to maintain normal gestation in the absence of ovaries and adrenals. There is also an apparent need of the hormone of the corpus luteum for normal gestation in the adrenalectomized rat.

DISCUSSION

The difference found by various investigators (1–6) in the ability of adrenalectomized rats to have normal pregnancy may be due in part to the strain of rats used. Rats from several colonies have been shown (23) to have different survival times and therefore varying degrees of susceptibility to adrenalectomy. Although a difference in the strain of rats used may be one of the causes for the successful pregnancies of the adrenalectomized animals in these experiments, it is not the only factor since recent work has demonstrated that the hormones of pregnancy and pseudopregnancy, particularly progesterone, benefit the adrenalectomized animal. It is also not necessary to postulate the presence of microscopic or unidentifiable cortical tissue in adrenalectomized rats to account for their having successful pregnancy. In the present study this has been demonstrated experimentally by showing that the life of the adrenalectomized rat is

maintained by pregnancy. The possibility that the beneficial effects were produced by functional residual adrenal tissue was ruled out by using animals in which the completeness of adrenalectomy was demonstrated by the ultimate death of the rats from adrenal insufficiency after the protective effects of pregnancy were removed.

Although it might be supposed that residual cortical tissue would keep the animal alive only during gestation and then degenerate, with resultant death of the animal, such is not the case; animals which have residual cortical tissue survive indefinitely. Abnormal estrous cycles may be found for a while in rats which have small amounts of residual cortical tissue, but after this tissue regenerates the animals are physiologically normal as judged by the regularity of their estrous cycles and their ability to have and raise normal young. The completely adrenalectomized rat, however, usually has regular estrous cycles up to the time adrenal insufficiency becomes acute. From that time until death, the animal is in continuous diestrus and will not mate. If the completely adrenalectomized rat becomes pregnant before adrenal insufficiency becomes acute, apparently the hormones of pregnancy benefit the animal to the extent of prolonging survival and allowing normal gestation.

In these experiments lactation was not supported, as was the life of these animals, by the hormones of pregnancy. The inadequate lactation shown by the failure of the litters to survive to weaning is further evidence of the absence of functional tissue of the adrenal cortex in these experimental animals, for it has been shown (24–26) that the cortical hormone is necessary for normal lactation.

The hormones of the placento-fetal complex were also found not to be adequate to permit successful gestation or to prolong survival of pregnant rats which had both ovaries and adrenals removed. There is an apparent need for the hormone of the corpus luteum to maintain pregnancy in the adrenalectomized rat.

These experiments confirm the findings (2, 8) that pregnancy prolongs the survival time of the rat after adrenalectomy, and indicates that the number of successful pregnancies was directly related to the time of adrenalectomy. Approximately 1% of the animals adrenalectomized before becoming pregnant, 40% adrenalectomized on the second day of pregnancy and 92% adrenalectomized on the 17–19th day of pregnancy bore normal litters. This suggests that the more advanced the pregnancy the better are the chances of having successful gestation.

These experiments also show that sesame oil injections do not affect the normal course of gestation in the adrenalectomized rat. These oil injections, although they were without effect on the survival of nonpregnant rats (table 2 A), did increase the number of successful pregnancies and prolong the survival of most of the oil treated females and their litters as compared with the untreated controls. The only exceptions to the general beneficial effects of sesame oil for adrenalectomized pregnant rats were found in the reduced survival time of the oil treated females which resorbed their young (table 2 B) and in the reduction in the percentage of successful pregnancies in the group adrenalectomized during the last quarter of pregnancy (table 2 C).

What role sesame oil plays in prolonging the survival time and increasing the percentage of adrenalectomized rats which have normal pregnancies is not determined at present. The factors involved in the beneficial action of sesame oil, and fractions of this oil, upon the rat after adrenalectomy and under various other experimental conditions is discussed elsewhere (27).

SUMMARY

Normal gestation and parturition can occur in the rat in the absence of tissue of the adrenal cortex, as judged by the ultimate death of the animal from adrenal insufficiency.

The number of successful pregnancies is directly correlated with the time of adrenalectomy. Approximately 1% of the animals adrenalectomized before mating, 40% of these adrenalectomized on the second day, and 92% of those adrenalectomized on the 17–19th day of pregnancy, bore normal litters but did not carry any of the young to weaning.

Normal estrous cycles were found in most of the animals having residual cortical tissue from sub-total adrenalectomy. If this tissue is given sufficient time to proliferate normal reproduction and growth of the offspring are found. None of the animals with residual cortical tissue died of adrenal insufficiency.

The placento-fetal complex alone in the animal after bilateral adrenalectomy and ovariectomy is not sufficient to maintain normal gestation or to prolong the survival time of the animal. The hormone from the corpus luteum apparently is also needed.

Injections of sesame oil increase the survival time of pregnant rats which deliver and their litters after adrenalectomy.

Sesame oil is not inert when used as a control oil in adrenalectomized rats which are pregnant.

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FATE OF OVARIES WHICH HAVE BEEN GRAFTED IN THE EAR FOR LONG PERIODS OF TIME[1,2]

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THE FIRST EVIDENCE of a dual hormone secretion from ovaries was probably collected by Lipschütz (1), and has been more recently studied and analyzed by an increasing group of workers (2–13). Some investigators (9, 14, 15) have found some androgenic activity as a result of injection of corpus luteum materials in castrated male rats although negative results were obtained in the mouse (3). Takewaki (7, 8) has studied the effect of ovarian androgens on the mouse adrenal, and also the effect of transplanting the adrenal. Zuckerman and Groome (11) recently made an interesting and thorough study of an intersexed pig, and discussed the presence of structures and possible correlation with physiology. Lipschütz (12) stated, relative to his work in the guinea pig, that experimentally produced ovarian androgenic activity was in no way correlated with histological changes suggestive of intersexuality. He further stated that medullary ovarian tubules and epithelial development does not necessarily mean androgenic activity. The discovery that androgenic activity of ovaries could be controlled (in mice at least) by temperature (3, 4), was a partial analysis of the subject. Beyond the temperature experiments only slight advancement has been made (5, 6, 10, 13) in an understanding of the mechanisms involved. Unpublished experiments by the author show that under special conditions thyroid activity can enter the picture of ovarian androgen formation, and the results of these experiments will be published soon.

During the course of many experiments it has been possible to study changes occurring in ovaries which have been grafted for long periods of time in the ears of castrated male mice. This paper deals with those findings.

DATA

The results taken from a total of 44 animals are to be considered here. The host animals carried their ovarian ear grafts for periods of from 305 to 605 days and were from 60 to 250 days old at the time of grafting. The actual age of the grafted ovaries at the time of killing the host animal varied from 405 to 665 days. All animals were kept in atmospheric temperature of 22 to 23°C.

While all animals did not undergo laparotomy after receiving an ovarian graft, the great majority of those which were laparotomized exhibited normal seminal vesicles and prostates up to six or more months after grafting. Castrate male accessories were found in only those few animals with obviously small and inferior grafts. No laparotomies were performed during the interval from six months after grafting until the animals were killed. In all cases one adrenal, a piece of thyroid, seminal vesicle and all of the grafted ovarian tissue was saved for histological study.

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[2] The mice used were all of the CHI inbred strain of Dr. L. C. Strong.

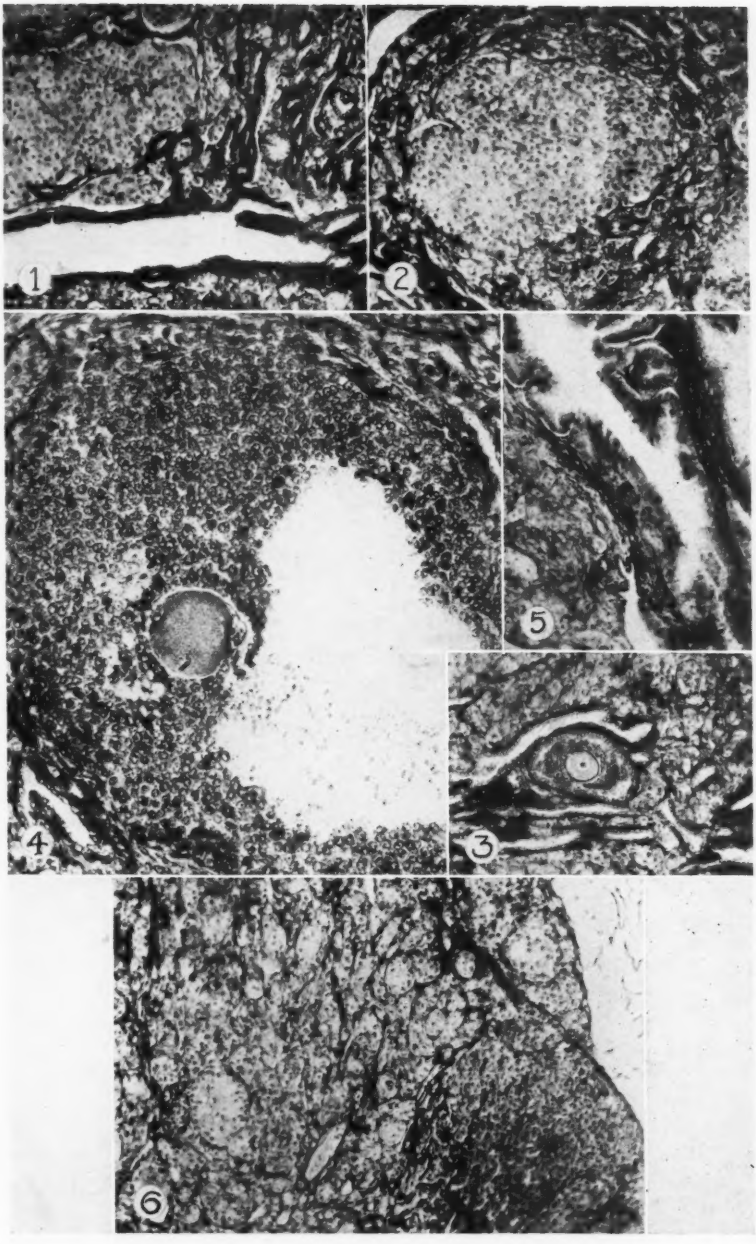

Fig. 1. Epithelial cords and clusters of lutein-like cells. ×110. Fig. 2. Probably a corpus luteum atretica, and containing some giant cells. ×110. Fig. 3. Normal follicle (young). ×110. Fig. 4. Ovum undergoing mitosis. Note mitosis in surrounding granulosa cells. ×210. Fig. 5. Fallopian tube. ×110. Fig. 6. Adrenal cortex. × 110. Note normal cortex alongside a large area of cortex with clusters of lutein-like cells. Compare with elements of ovary in figures 1, 2, 3 and 5.

Thyroid tissue. The thyroid tissue removed from these mice presented in every case and in all respects a normal histological appearance. Whether or not function of the thyroid gland affected the results obtained in these experiments remains a question. If the histological picture of thyroid tissue is a true index of function, then we need not consider it further in these results.

Seminal vesicle tissue. The histological picture as obtained in normal and castrate type seminal vesicles is, of course, well known. It was used here as an end point index of androgenic activity from the grafted ovarian tissue. The seminal vesicles were classed as follows: normal; fairly normal with granular epithelium but lacking in secretion; of near castrate size and structure; and of a true castrate type.

In all but 5 cases the male accessories were of a typical castrate type when the animals were killed. In each of these 5 instances the ovaries had been grafted for a total of 305 days each and were 100 days old at the time of grafting. Hence the ovaries were actually 405 days of age at the time the host was killed. In each case the host was 430 days of age at killing. The discrepancy between the age of the host and the age of the grafted ovary is thought not to be significant.

Ovarian tissue. The ovarian tissue was carefully examined for type and degree of development, as follows: cords of connective tissue and epithelial cells; giant cells; picnosis; follicles; ova; and lutein or lutein-like tissue.

Relative to cord development one thing is quite noticeable. The cords were predominantly of connective tissue in those ovaries which had been grafted for 350 to 600 days. In those ovaries which had been grafted for shorter periods of time the cord arrangement was composed more of epithelial cells, although the overlapping of types was considerable. The epithelial cords, it is believed, have proliferated directly from the germinal layer of the ovary, and in many places can be seen to be continuous with the germinal epithelium (fig. 1). These cords do not appear to be a result of medullary hypertrophy of embryonic testis, though at times they do assume a tubular appearance, but apparently by chance alone. At all times during the life of a grafted ovary there is present varying amounts of connective tissue stroma. As the graft becomes older the epithelial elements apparently undergo picnotic involution and leave behind a proportionately increasing quantity of connective tissue, thus giving rise to the connective tissue cords.

A type of giant cell is found throughout the series, in greater or lesser numbers, and apparently without correlation to age (beyond 300 days) or function (fig. 2). There is some evidence that such giant cells may be formed by a coalescence of two or more epithelial cells at some time during or prior to picnotic involution. There is no correlation between the number of these cells present and the age or physiology of the graft in these experiments. We have not described these cells as being found in earlier experiments, because they were so few in number. They increase in number somewhat in long time grafts, and probably more are derived from the lutein-like tissue than from the epithelial cords and germinal epithelium.

There are very few follicles present which have a normal appearance. However, a few normal follicles were present and contained ova in either resting stages or in mitosis (fig. 3, 4). Mitosis appeared in an ovum of an ovary of 405 days of age which had been grafted into a castrated male for 305 days. The host was 100 days of age at the time of grafting.

Some follicles appeared to be undergoing atretic luteinization, and lutein-like tissue appeared in quantities throughout most of the grafts (fig. 2).

In one case of a 335-day graft a portion of the fallopian tube was present from grafting, and still had active and normal epithelium, signifying the presence of some

estrogenic activity in the graft (fig. 5). The graft (tube and ovary) was 395 days old, and grafted in to a host which was 525 days old at the time it was killed.

DISCUSSION

In the experiments described here ovaries which had been grafted from 305 to 605 days were studied. In 5 of the 44 cases studied the development of the seminal vesicles proved the presence of some male hormone at the time the animals were killed. In each of these cases the graft had been present for 305 days. These data seem to show that the androgenic activity of grafted ovaries (in mice) will not continue at threshold levels at a point of ovarian age beyond about one year. This period of time has a striking correlation with the period of greatest fertility in the normal breeding female. In the intact male the testes will maintain threshold amounts of male hormone for periods of time considerably longer than the period of fertility in the female. When it was first discovered that ovaries had physiological amounts of androgens present it was thought that perhaps the interval of androgenic activity might be longer than the period of fertility. These data fairly completely disprove that supposition.

In so far as epithelial and connective tissue cords were found in a greater or lesser degree throughout the entire series, it likewise seems that they must have no special function as regards androgenic activity of the grafts. In earlier work mention was made of the presence of lutein-like cells. The work of several investigators (9, 10, 14, 15) has shown that progesterone has some androgenic activity. Therefore the assumption was that perhaps the presence of this peculiar cell type might logically explain the male hormone physiology of ovaries under the conditions of these experiments. In the experiments reported here lutein-like cells were found throughout the series. If in the younger grafts an androgenic physiology is demonstrated, and if in long time grafts the androgenic activity disappears with no significant or constant change occurring relative to lutein-like cells, then it may follow that male hormone is not necessarily correlated with their presence. If their presence is necessary to androgenic activity, then the disappearance of male hormone physiology may be correlated with structural change which is not visible. Further, it is possible that they are necessary to androgenic activity, but that they are only a link in a chain of reactions. If such is the case then the link that fails remains to be found. The anterior lobe of the pituitary is not a likely party to the failure, as it maintains a fairly normal female physiology in the grafted ovaries for periods of time greater than that of male physiology. Further, it maintains testes in the male for likewise greater periods of time. It is possible that in the normal male there exists some peculiar interaction of testis and anterior lobe which fails abnormally early in the life of the animals here considered. In these cases, of course, it would be an ovarian androgen—anterior lobe interaction instead of a testis-pituitary complex.

In as much as the thyroid histology remains normal in all cases of this series it seems that we must, for the time being at least, consider it normal in function, and thus dismiss it.

The adrenals show no consistent histological picture or change correlated with the presence or absence of androgenic activity. There is, however, one point of interest regarding the adrenals in these experiments. It relates to certain cells of the cortex in a few cases which have a nearly identical histological appearance with certain cells found in the grafted ovaries (fig. 6). The only significance attached to this finding is that with a common regional embryonic origin of the ovary and adrenal, the two glands may obtain certain cells in common. The histological study of the

normal mouse adrenal and ovary does not substantiate this statement. However, it may be that the special conditions of these experiments serve to clarify the similarity or identity of these cells. It is believed that the similarity (or identity) of these cells has no special significance relative to androgenic activity, but nevertheless they remain of histological interest.

The presence or absence of follicles bears no correlation with the physiology described. All stages of follicular development can be found. Thus it is to be noted that the mouse apparently does not fall in the class of animals with the rat relative to masculinization or feminization of the pituitary as described by Pfeiffer (16, 17). Although these experiments have been of an entirely different nature than those described by Pfeiffer, the above assumption seems logical.

Lampton and Miller (13) raise the question as to whether an androgen-like substance is elaborated by the ovary, or whether the response obtained may be the androgen stimulating ability of one of the female hormones. This question arises from the results obtained by Greene, Burrill and Ivy (15), but their experiments unfortunately offer no answer. However, their findings are of much interest relative to temperature effects on an ovary. It must also be remembered that (3) progesterone injections in castrate male mice failed to give an androgenic response, although positive results have been obtained in the rat (9, 14, 15). Furthermore (12) the histological picture found in guinea pig ovaries did not correlate with any androgenic function.

It remains, then, that the androgenic activity of the mouse ovary probably comes by the way of some peculiar metabolic chemism as a result of temperature change in the gland.

The length of time during which an ovary can produce threshold amounts of androgens is probably controlled by the inherent genetic constitution of ovarian tissue. If this be the case, then it can only be hoped that at some future date it may be more fully understood.

SUMMARY

The data presented here lead to the belief that androgenic activity of ovaries in mice is not of necessity correlated with any histological structure of the gland. Likewise no explanation is offered by the structure of any other gland studied. It is reasoned that such function is not likely to come from the pituitary. The interval of ovarian androgenic activity is a likely genetic inherent quality of the ovary. It is suggested that the male hormone action of the ovaries in these experiments is a metabolic chemism of the gland due to temperature.

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FRACTIONATION STUDY OF THE RESIDUAL OVARY

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THE RECOGNITION, many years ago, of the ovary as an organ of internal secretion has been followed by numerous preparations of extracts from ovarian tissue. These extracts when administered to female animals were expected to introduce characteristic changes in accessory sex organs. The greatest obstacle encountered in the earlier work was the lack of a rapid, quantitative method of assay. Observations made by Stockard and Papanicolaou (1) of the changes in the epithelium of the vagina, and the hypertrophy of the uterus have led to such a rapid quantitative method of assay. Long and Evans (2), Coward and Burn (3), D'Amour and Gustavson (4), and many others were helpful in refining and adapting the vaginal smear method as a rapid, quantitative method of assay of extracts containing estrogenic compounds.

In the study of the ovary, most of the work has been done on the follicular fluid, on the whole ovarian extracts, and on the residual ovary. In table 1 below are listed some of the workers in the field, together with their yields of estrogenic substances from these fractions of the ovary.

TABLE 1. YIELDS OF ESTROGENIC FACTORS FROM OVARIAN TISSUE

Observer	Date	Estrogenic content and source
Allen, Meyer (9)	1933	100 R.U./kg. ovary tissue
D'Amour, Gustavson (10)	1933	950 R.U./liter follicular fluid
D'Amour, Gustavson (10)	1933	65 R.U./kg. residual tissue
Marlow, Groetsema (11)	1935	276 R.U./kg. of residual ovary
Ralls, Jordan (5)	1926	1000 R.U./liter follicular fluid
Sealey, Marlow (12)	1936	300 R.U./kg. of residual ovary
Thayer, Doisy (6)	1928	933 R.U./liter follicular fluid
Thayer, Doisy (6)	1928	450 R.U./kg. whole ovary
Thayer, Doisy (6)	1928	168 R.U./kg. residual ovary

Table 2 shows some of the properties of crystalline estrogenic compounds obtained by various workers.

TABLE 2. PROPERTIES OF ESTROGENIC HORMONES

Name	M.P.	Empirical formula	Activity	Discoverer
			R.U./mg.	
Estrone	253	$C_{18}H_{22}O_2$	8,000	Doisy (14), Butenandt (15) 1930
Estriol	273	$C_{18}H_{24}O_3$	1,000	Doisy (16), Butenandt (17) 1931
Estradiol	170	$C_{18}H_{24}O_2$	40,000	MacCorquodale, Thayer, and Doisy (18), 1935.
	95.1	$C_{20}H_{41}O_2N$	100,000	Andrew and Fenger (19) 1936

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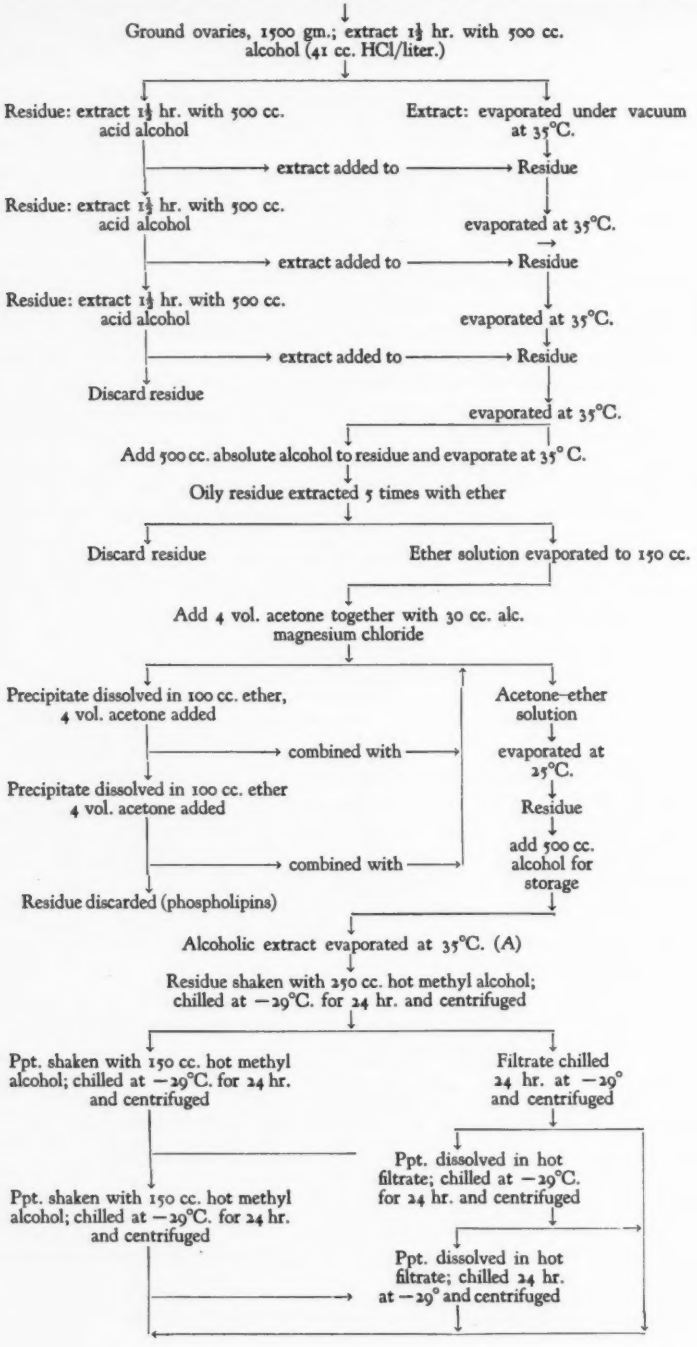
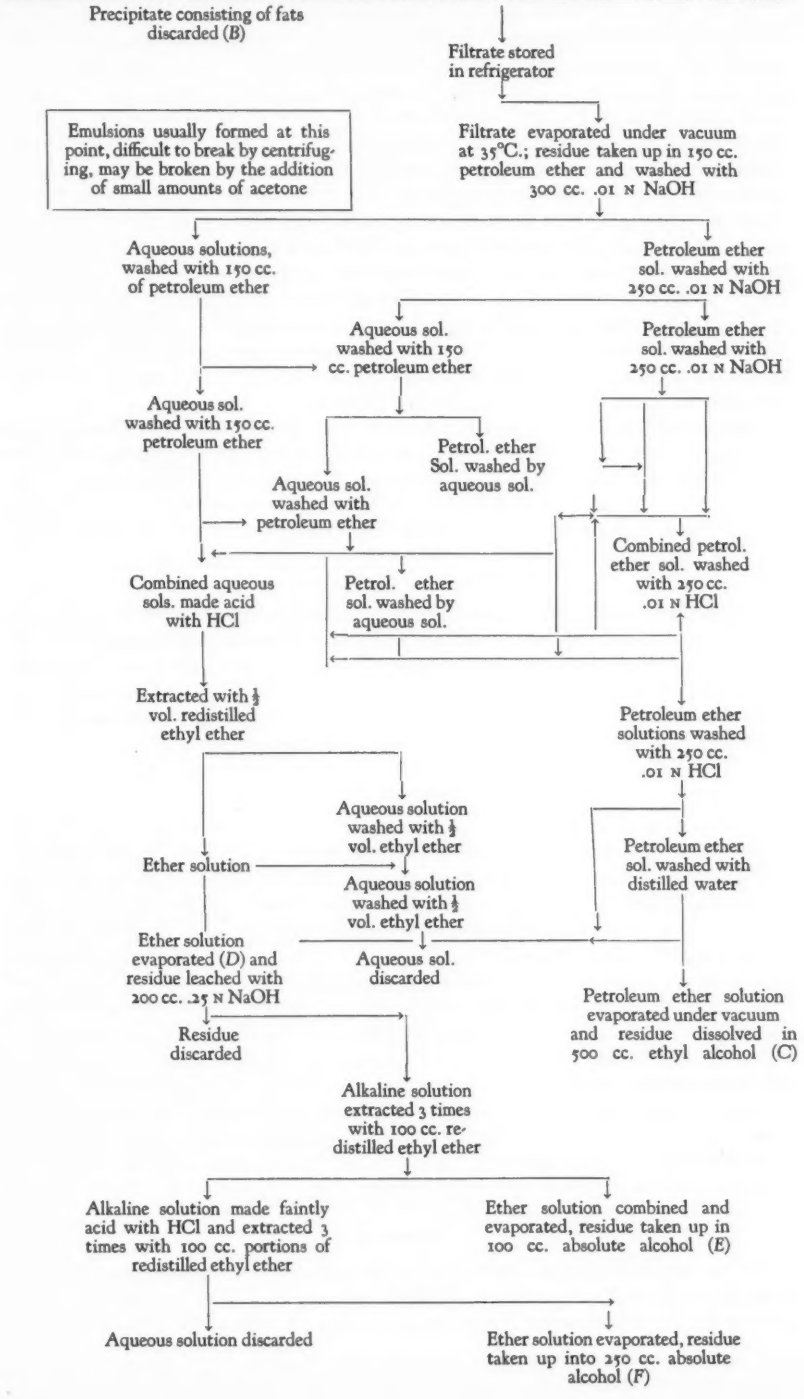
[1] Submitted in partial fulfillment of the requirements for M.S. degree, 1936.

SCHEMATIC DIAGRAM OF SEPARATION OF ESTROGENIC FACTOR FROM RESIDUAL OVARIAN TISSUE


Ground ovaries, 1500 gm.; extract 1½ hr. with 500 cc. alcohol (41 cc. HCl/liter.)
Residue: extract 1½ hr. with 500 cc. acid alcohol
Extract: evaporated under vacuum at 35°C.
extract added to
Residue
Residue: extract 1½ hr. with 500 cc. acid alcohol
evaporated at 35°C.
extract added to
Residue
Residue: extract 1½ hr. with 500 cc. acid alcohol
evaporated at 35°C.
extract added to
Residue
Discard residue
evaporated at 35°C.
Add 500 cc. absolute alcohol to residue and evaporate at 35° C.
Oily residue extracted 5 times with ether
Discard residue
Ether solution evaporated to 150 cc.
Add 4 vol. acetone together with 30 cc. alc. magnesium chloride
Precipitate dissolved in 100 cc. ether, 4 vol. acetone added
Acetone–ether solution
combined with
evaporated at 25°C.
Precipitate dissolved in 100 cc. ether 4 vol. acetone added
Residue
combined with
add 500 cc. alcohol for storage
Residue discarded (phospholipins)
Alcoholic extract evaporated at 35°C. (A)
Residue shaken with 250 cc. hot methyl alcohol; chilled at −29°C. for 24 hr. and centrifuged
Ppt. shaken with 150 cc. hot methyl alcohol; chilled at −29°C. for 24 hr. and centrifuged
Filtrate chilled 24 hr. at −29° and centrifuged
Ppt. dissolved in hot filtrate; chilled at −29°C. for 24 hr. and centrifuged
Ppt. shaken with 150 cc. hot methyl alcohol; chilled at −29°C. for 24 hr. and centrifuged
Ppt. dissolved in hot filtrate; chilled 24 hr. at −29° and centrifuged

SCHEMATIC DIAGRAM OF SEPARATION OF ESTROGENIC FACTOR FROM RESIDUAL OVARIAN TISSUE—*Continued*


Precipitate consisting of fats discarded (B)
Filtrate stored in refrigerator
Emulsions usually formed at this point, difficult to break by centrifuging, may be broken by the addition of small amounts of acetone
Filtrate evaporated under vacuum at 35°C.; residue taken up in 150 cc. petroleum ether and washed with 300 cc. .01 N NaOH
Aqueous solutions, washed with 150 cc. of petroleum ether
Petroleum ether sol. washed with 250 cc. .01 N NaOH
Aqueous sol. washed with 150 cc. petroleum ether
Petroleum ether sol. washed with 250 cc. .01 N NaOH
Aqueous sol. washed with 150 cc. petroleum ether
Petrol. ether Sol. washed by aqueous sol.
Aqueous sol. washed with petroleum ether
Combined petrol. ether sol. washed with 250 cc. .01 N HCl
Combined aqueous sols. made acid with HCl
Petrol. ether sol. washed by aqueous sol.
Extracted with ½ vol. redistilled ethyl ether
Petroleum ether solutions washed with 250 cc. .01 N HCl
Aqueous solution washed with ½ vol. ethyl ether
Ether solution
Petroleum ether sol. washed with distilled water
Aqueous solution washed with ½ vol. ethyl ether
Ether solution evaporated (D) and residue leached with 200 cc. .25 N NaOH
Aqueous sol. discarded
Petroleum ether solution evaporated under vacuum and residue dissolved in 500 cc. ethyl alcohol (C)
Residue discarded
Alkaline solution extracted 3 times with 100 cc. redistilled ethyl ether
Alkaline solution made faintly acid with HCl and extracted 3 times with 100 cc. portions of redistilled ethyl ether
Ether solution combined and evaporated, residue taken up in 100 cc. absolute alcohol (E)
Aqueous solution discarded
Ether solution evaporated, residue taken up into 250 cc. absolute alcohol (F)

Groetsema and Marlow (11) observed the effects of the extracts from residual ovaries upon immature female rats in causing vaginal introitus, and hypertrophy of the uterus. They also observed that the estrogenic compound did not affect the normal heat cycle of the young rats. Marlow (13) in a later paper showed the effects of residual ovarian extracts upon the estrous cycle of adult castrates and normal female albino rats. A dosage sufficient to cause vaginal introitus in young females, also caused estrus in ovariectomized rats. This dosage or even twice this dosage when injected daily over a period of 25 days did not affect the estrous cycle of either the old or young normal rats. It was also observed that the blood serum of uncastrated animals that had been injected with residual ovarian extracts, and whose cycle had not been disturbed, contained enough of the active principle per cc. to cause cornification of the epithelial cells in the vagina of the ovariectomized animal. Estrone injected in the castrated females showed estrus, but their blood serum did not contain sufficient amount of estrone to cause estrus in an ovariectomized rat. Both groups of animals were killed when the vaginal smear indicated diestrus.

Because of these observations, it was decided to continue the study by chemical separation. A scheme of separation was outlined by which the estrogenic compounds could be concentrated in their active fractions, and quantitative studies made of the activity where found. The method used is illustrated in a schematic form which is given in detail in order that it may be followed exactly by a worker wishing to do so.

MATERIALS AND METHODS

Ovaries from which all the possible follicular fluid had been aspirated, were used as the source of the residual ovaries for this study. (The authors are especially indebted to Dr. E. A. Doisy for these ovaries.) The extracts were made in an extractor similar to one described by Groetsema and Marlow (11). The object of this study was to make extracts, and to assay each fraction obtained during the process of purification for estrogenic activity. The outline gives exact methods employed.

Biological Assay

The method of assay used throughout the experimental work was essentially the technic of Coward and Burn (3) and Loewe and Lange (7). The animals selected were young mature albino rats, that had been castrated 2 weeks previous to their use. Only the rats showing a diestrous smear for 5 consecutive days were used. Each assay was made by a single subcutaneous injection of a series of varying amounts of the fraction incorporated into olive oil usually equivalent to 1, 3, 5, 8, 10, 20, 50 and sometimes of 100 gm. of fresh tissue. If more than 50 gm. equivalent were necessary to give an estrous smear, the estrogenic factor was considered negligible for this study. Vaginal smears were made at the time of injection, for a control smear, and at intervals of 24, 36, 48, and 60 hours, after a single subcutaneous injection. Estrus was accepted only upon complete disappearance of the leucocytes, and the appearance of only masses of non-nucleated epithelial cells, as described by Long and Evans (2). The amount which caused 10 rats, or 50% of the injected rats to respond was taken as the rat unit. The chart prepared by D'Amour and Gustavson (4) is helpful in determining dosages in the event that the number responding is not just 50% of those injected.

DISCUSSION

In the schematic diagram, points in the procedure at which assay were made are lettered to aid in the description of the substance assayed. The estrogenic compounds that have been isolated from various sources are estrone, estriol and estradiol.

However, no attempt was made to separate estradiol from the other as it is thought to be the principal hormone in follicular fluid which had been removed from the ovarian tissue. Since it requires 180 or more grams of fresh pig ovary to furnish the equivalent of one rat unit of progesterone (20) it was not separated as single units when the lower dosages were used. Previous assay of the ovary for estrogenic content showed no detectable difference whether the corpora lutea were removed or not removed. At *A* all activity corresponding to the combined residual ovarian extracts was found. *B* represents a separation of neutral fats, largely, and dosages up to 100 gm. equivalent gave negative estrogenic response. C is a fraction expected to contain progesterone, which exhibited no estrogenic characteristics up to 25 gm. equivalent of fresh tissue and which showed no inhibition of estrous cycle of normal female rats in dosages of 50 gm. and more *D* still retains the activity of the residual ovarian extracts, as in A, with a loss of less than 8% activity. The fraction designated by *E* is the estriol or estradiol which assayed about 1 R.U. per 15 gm. of tissue, while the fraction *F* represents estrone. The latter assayed 1 R.U. for 40 gm. equivalent. In other words the estrogenic fractions were carried over to the fractions containing estriol, estradiol and estrone according to the available methods of separation. The estriol or estradiol fraction is more potent in its effect upon castrate female rats than the estrone fraction.

SUMMARY

A method by which each estrogenic fraction may be separated completely from the ovary is outlined.

Of the 6 fractions only the 2 containing estriol (or probably estradiol) and estrone have any assayable estrogenic activity. The estrone fraction possesses less activity than the estriol fraction.

Estradiol is thought to be the estrogenic compound in follicular fluid, but as the follicular fluid was not included, no attempt was made to isolate it as a separate factor.

A biological examination of the fractions separately, and of the ovarian extract would be helpful in identifying, finally, these estrogenic compounds (8).

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THE EFFECT OF HYSTERECTOMY UPON THE OVARY

WITH REFERENCE TO A POSSIBLE HORMONAL ACTION OF THE ENDOMETRIUM UPON THE OVARY

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THE PROBLEM of the fate of the ovaries following hysterectomy is still under discussion. Many believe that the removal of the uterus initiates atrophic changes in the ovaries with subsequent onset of menopausal symptoms. Others maintain that the uterus has no direct influence upon ovarian function, and that whatever changes are brought about are subsequent either to mechanical trauma, or to interference with ovarian blood supply following operation.

Early workers (1–5) established the fact that abnormal changes occur in the ovary of laboratory animals following hysterectomy. Others (6, 7, 8) attempted to prevent the development of such changes by transplantation of endometrial tissue. Within recent years the suggestion has appeared that the uterus elaborates a hormone with a definite action upon the ovary (9, 10). In an experimental study on rabbits, Sessums and Murphy (11) concluded that a degenerative effect upon the ovaries is produced by removal of the uterus and that autotransplantation of endometrial tissue may limit the extent of the inhibitory and degenerative changes which result from hysterectomy.

In the human being, Fluhmann (12) studied several sections of both ovaries removed from a young woman one year following total hysterectomy. No evidence of recent corpus luteum formation was found, the ovaries contained a large number of atretic cystic follicles. Tamis (13) studied the effects of hysterectomy upon ovarian function in 18 women by means of gonadotropin and estrone determinations in the urine. Greenhill (14) states: "No one has yet definitely proven the value of uterine extracts nor has anyone performed controlled experiments with these extracts comparable to the experiments performed with the hormones obtained from the ovary, pituitary, pancreas, etc."

The present study is based upon the findings in the ovaries of a series of rabbits, which may be grouped as follows.

Group A. Hysterectomy alone

Group B. Hysterectomy plus the injection of an endometrial extract for a 3-month period

Group C. Hysterectomy plus autotransplantation of endometrial tissue

Group D. Controls, normal animals

Group E. Normal animals injected with endometrial extract and killed after 3 months

All animals used were young adult females of approximately the same age and breed, weighing between 2500 and 3000 gm.

The endometrial extract used was prepared from fresh endometrial tissue removed from non-pregnant cow uterus immediately after killing. The endometrium was removed by scraping, and was immediately placed in a vacuum dessicator and de-

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hydrated at a temperature not exceeding 39° C. The desiccated substance was then thoroughly extracted in a Soxhlet apparatus with organic solvents. The fractions were combined, the solvent removed, and the extract dissolved in sesame oil; 1 cc. of oil contained the extract of 1.25 gm. of endometrium.

Histological examination of the rabbit ovaries by one of us, (L.M.) gave an immediate impression that there was a striking difference between those rabbits which were subjected to complete hysterectomy, and those, which, in addition to removal of the uterus, were treated with endometrial extract by injection. The changes were noted in the follicular apparatus and the interstitial gland. Before the histological findings are presented, it should be stated that not all the changes shown as typical of one group were entirely absent in the other groups. They were selected, however, as the predominant type characteristic of that particular group.

An analysis of the histological changes follows.

TABLE 1. GROUP A—HYSTERECTOMY (No. of animals 11; average time of experiment 17 weeks)

Animal No.	Follicles	Corpora lutea	Interstitial gland
3	Prolif. ++	o	Moderate dev. ++
4	Prolif. ++	o	Moderate dev. ++
5	Atrophy o	o	Moderate dev. ++
10	Atrophy o	++	Atrophy o
11	Atrophy o	o	Atrophy o
12	Prolif. ++	o	Atrophy o
13	Prolif. ++	o	Moderate dev. ++
14	Atrophy o	o	Moderate dev. ++
18	Prolif. +	Small	Regression
19	Prolif. ++	Atretic	Regression
20	Atrophy o	++	Regression

Interstitial gland. (*a*) moderately developed, 5 cases (3, 4, 5, 13, 14); (*b*) tendency to atrophy, smaller cells, fibrosis of intercellular substance, 3 cases (10, 11, 12); (*c*) very pronounced regression, which followed a functional hypertrophy of the interstitial gland, 3 cases (18, 19, 20); in one of these (20) a fragmentation of the cells was noted (Fig. 1).

Follicular apparatus: (*a*) moderate proliferation of follicles (without corpora lutea) 4 cases (3, 4, 12, 13); (*b*) distinct atrophy (without corpora lutea) 3 cases (5, 11, 14)

Corpora lutea: (*a*) true (degenerating) corpus luteum plus a distinct tendency to atrophy of the follicular apparatus, 2 cases (10, 20) observed for 23 weeks; (*b*) very small corpus luteum plus very low, but distinct proliferation of the follicles, 1 case (18) observed for 23 weeks; (*c*) an atretic corpus luteum plus distinct proliferation of follicles, 1 case (19) observed for 23 weeks.

The last 2 cases present a certain degree of follicular hyperactivity resulting, however, in the formation of abnormally small, or atretic corpora lutea. It is interesting to note that the interstitial gland apparently reflected these changes.

Conclusions for animals subjected to hysterectomy alone. The animals, after complete removal of the uterus show: (*a*) atrophy of the follicular apparatus, the changes of precocious regression were particularly marked in 5 cases (of 11 studied); (*b*) the theca interna, even in the youngest follicles, was markedly fibrous (fig. 2); (*c*) a tendency to abnormal and exaggerated functional activity of the follicular apparatus, which did terminate, however, in the formation of normal or approximately normal corpora lutea, was noted in 4 cases; atretic corpora lutea (fig. 3) and paradoxical luteinization of the theca in atretic follicles (fig. 4, 5) were also found; (*d*) the interstitial gland showed a tendency to compensatory hypertrophy which terminated in exaggerated premature degeneration of the cells (as found in 3 cases); the cells were hydropic, vacuolated, and distended; the nucleus shrank due to the pressure of the liquefied contents of the cytoplasm (fig. 1).

TABLE 2. GROUP B. HYSTERECTOMY PLUS INJECTION WITH ENDOMETRIAL EXTRACT. NUMBER OF ANIMALS 10. THE DOSE OF THE ENDOMETRIAL EXTRACT GIVEN WAS 0.5 CC. DAILY, EQUIVALENT TO 0.0125 GM. OF DRIED EXTRACT. THEY WERE INJECTED 5 TIMES WEEKLY FOR A PERIOD OF 12 WEEKS.

Animal No.	Follicles	Corpora lutea	Interstitial gland	
6	+++	+++	Collapse	o
7	+++	+++	Beginning atrophy	o
8	+++	o	Fibrosis	o
9	++++	o	Well developed	+++
15	++++	++++	Marked fibrosis	o
16	++	o	Moderate fibrosis	+
17	+++	++	Well developed	+++
21	+++	++++	Collapse	o
22	+	++++	Collapse	o
23	o	++	Collapse	o

Interstitial gland. (*a*) well developed, 2 cases (9, 17); (*b*) regression from the previous state of good functional development, 2 cases (22, 23) (fig. 6); (*c*) partial collapse of cells, 2 cases (6, 21); (*d*) beginning atrophy and fibrosis, 1 case (7); (*e*) moderate fibrosis, 1 case (16); (*f*) marked fibrosis, 2 cases (8, 15).

Follicular apparatus: (See fig. 7, 8). If the highest degree of follicular activity is graded as 4 + then the average for this group of animals was 3 +. Two cases (9, 15) were 4 +; one case (16) was 2 +; only one case showed regression (22); and only one case showed marked atresia (23).

Corpora lutea: Seven of 10 animals showed the presence of corpora lutea. The corpora lutea were of normal size (fig. 9) and moreover, in some cases, showed a distinct evidence of hypertrophy very similar to that found in pregnancy. It is significant that in this group of animals the signs of regression of the corpora lutea were scant; when present, however, they were characterized by a paradoxical combination of excessive fibrosis of the nucleus and large areas of well preserved granulosa lutein cells. These findings indicated that the regression was very slow or retarded. Three animals (8, 9, 16) did not show any corpora lutea. One of them (9) had a fairly well developed interstitial gland. Two (8, 16) showed fibrosis of the interstitial gland. A marked lymphangiectasis was also noted in 2 of the cases (8, 9).

Conclusions to this group. (*a*) endometrial extract serves to regulate the process of follicular proliferation; it does not prevent the spontaneous formation of corpora lutein the rabbit ovaries after a hysterectomy; if a corpus luteum is formed, however, it is protected by the endometrial extract, which inhibits the process of its regression, apparently increasing the actual longevity of the corpus luteum; (*b*) the interstitial gland was in a state of paradoxical hypertrophy as found in the previous group.

In the animals which were injected with endometrial extract, the interstitial gland showed active proliferation of cells, which did not assume, however, the exaggerated proportions noted in the previous group. Here the interstitial gland was distinctly cellular, revealing a gradual regression and fibrosis. The interstitial gland of the animals used as controls also revealed considerable fibrosis, which is apparently a normal finding resulting from the fact that the animals were kept in a state of constant sexual rest.

Because the endometrial extract regulates the process of follicular proliferation, it protects the functional elements of the ovary, and retards the process of atrophy.

Group C. Hysterectomy plus autotransplantation of endometrial tissue. Five animals were studied by means of implanting pieces of endometrium, 2 to 3 mm. in diameter, between the peritoneum and muscular coats of the abdominal wall. Three months later the animals were killed, and both the implants and the ovaries were carefully studied. The results were varied and striking, and will be published in detail in a subsequent paper. However, it may be said at this time, that the functional

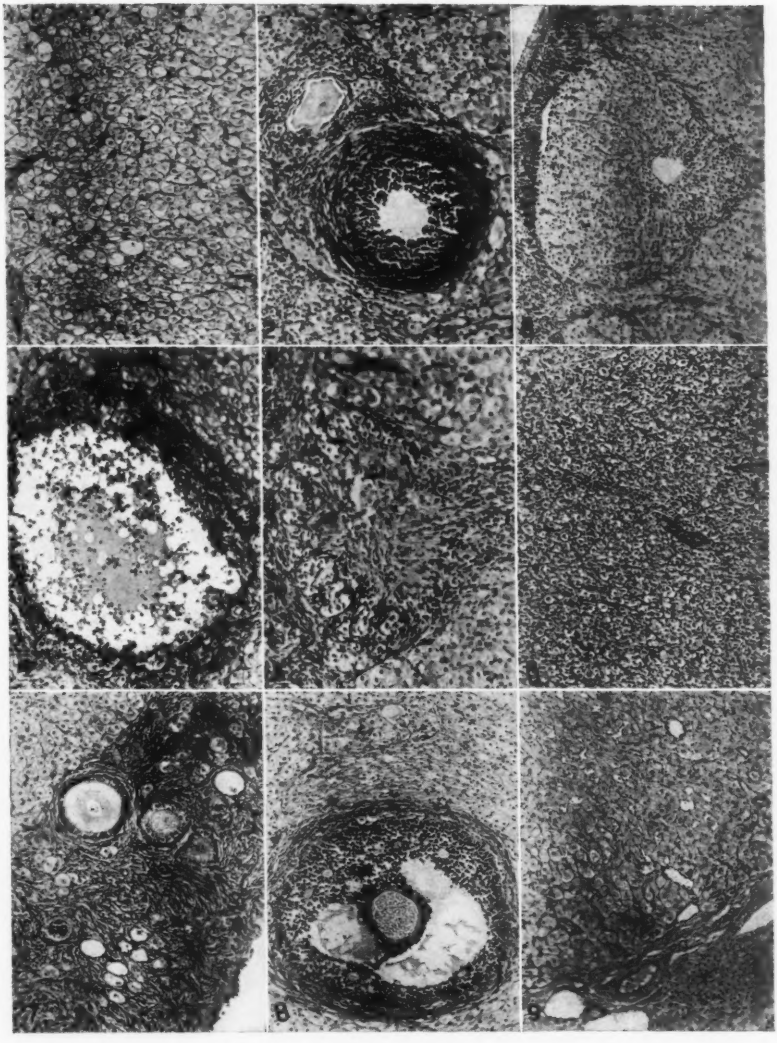

Fig. 1. Interstitial Gland. Paradoxical hypertrophy of the cells. Loss of luteinized substance. Shrinking of the nuclei. Vacuolization, irregularities in size, and shape of the individual cells. Fig. 2. Young ripening follicles. Fibrosis of theca, and precocious regression. Fig. 3. Corpus luteum. Atresia and beginning regression. Fig. 4. Graafian follicle. Atresia. Granulosa cells suspended in the follicular fluid. Paradoxical luteinization of theca interna. Fig. 5. Follicle in state of advanced organization. Formation of scar. Marked luteinization of theca interna. Fig. 6. Injected animal. interstitial gland. Beginning shrinking of the cells. The nuclei are more or less prominent, well preserved. The cytoplasm is not liquefied. Fig. 7. Injected animal. Ripening follicles. Note absence of regressive changes. Fig. 8. Injected animal. A normal graafian follicle. The zone of the theca interna is narrow and more or less cellular. Fig. 9. Injected animal. Large corpus luteum completely vascularized. The granulosa cells are well preserved and show beginning collapse in a few areas. Retarded regression of a corpus luteum.

character of the transplants were reflected by corresponding changes in the ovary. For example: a transplant that showed definite secretory changes, also revealed marked functional activity of the ovary with formation of true corpora lutea.

Group D. Controls, normal animals. Animals which were used as controls were of the same strain and breed as those used in our experiments. They were killed at the age of 5 months. A certain allowance should be made in the evaluation of histological findings because the animals were isolated and subjected to an unusually long sexual rest lasting many months.

Follicular apparatus. The follicles were found in practically every possible phase of maturation. There was no evidence of premature atrophy. A number of atretic follicles (cysts) were found. Corpora lutea were lacking.

Interstitial gland. The interstitial gland was moderately cellular. Neither hypertrophy, nor regression could be noted. The luteinization of stroma cells was limited to certain areas only. Moderate fibrosis of the cells of the interstitial gland, indicating apparent lack of sexual activity, was noted.

Group E. Normal animals injected with endometrial extract and killed after 3 months. Number of animals, 11; period of injection, 12 weeks. The purpose of this controls series was to determine whether the endometrial extract caused any change in the ovaries of rabbits in which the uterus was left intact. Histological examination failed to show any appreciable difference between this group and the previous normal control animals. The follicles were found in various stages of maturation with no corpora lutea present.

CONCLUSIONS

Removal of the uterus in the rabbit produces changes in the ovary which can be demonstrated within 3 months of time of operation. These changes consist of a precocious atrophy of the follicular apparatus, abnormalities in the growth of the follicles, atypical development and degeneration of corpora lutea, and compensatory hypertrophy in the interstitial gland terminating in cellular degeneration.

These changes are modified by the administration of endometrial extract prepared from cow's endometrium, which seems to regulate the process of follicular proliferation, protect the functional elements of the ovary, and retard the process of atrophy.

The results of these experiments are sufficiently encouraging to justify further study of the action of the endometrial extract in anthropoids. This is being carried out at the present time.

Appreciation is expressed to Charles F. Longfellow, M.D. of G. W. Carnrick Co., Newark, N. J., for supplying the endometrial extracts used in these experiments.

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EFFECTS OF ESTROGEN AND PROGESTERONE UPON THE GENITAL TRACT OF THE EWE[1]

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STERILITY in ewes has not received a great deal of study, probably because of their relatively small individual value and the customary method of handling them. A large number of ewes, however, are marketed annually after failing to produce lambs for one, or in the case of very valuable ewes, for two years.

Careful studies have been made of the changes occurring in the genital tract of the ewe during the different phases of the estrual cycle. Indications of the effects of the two hormones of the ovaries, estrogen and progestin, may be obtained from these studies, but their effects cannot be separated. A clearer understanding of the function of each of these hormones would be of decided value if it could be applied to the correction of sterility and the maintenance of fertility in the ewe. This article reports a study in which an attempt has been made to determine, more accurately, the roles played by these two endocrine substances in the reproductive tract.

The only available report of studies made of the effects of estrogens upon the genital tract of castrate farm animals is given by McKenzie and Terrill (1). Three ovariectomized ewes were included in their study. Two of these were killed at the end of artificially induced estrus produced by injections of estradiol benzoate (1000 R.U. given in 5 injections of 200 R.U. at 6-hour intervals). The two ewes came in heat 24 and 36 hours after the first injection. The third ewe received no injection. In the uninjected castrate, regression was found in each of the genital organs. Vaginal, cervical, and fallopian tube epithelial linings were all higher in the treated ewes. Injections of estradiol also caused the lumina of the uterine glands to become much larger.

Histological changes in the genital tract of the ewe during the estrual cycle have also been reported (1–5). Vaginal epithelium was highest during estrus. Cornification, though variable, was usually noted in late estrus and metestrum. Leucocytes were found to be most numerous in the epithelium during the middle and latter part of the cycle.

Reports of changes in the cervix are not in agreement. Casida and McKenzie (2) noted increased secretory activity in the 9 to 11-day stages. Grant (4) found the secretion to be continuous throughout the cycle and found no cytological evidence of differences in the intensity of secretion. He concluded, however, that secretion must be greater during estrus to account for the large amount of mucus found at that time. Cole and Miller (5) also reported that mucus secretion was greater during proestrum and estrus. The increased secretion was indicated by the distention of the secretory cells with mucus and the tendency of their nuclei to become flattened against the base of the cells. McKenzie and Terrill (1) reported excretory activity of the columnar epithelium of the cervix at all stages of the cycle. Their observations indicate the tendency for the secretion to accumulate in the cells during the luteal phase of the cycle and to be eliminated during late luteal and proestrual stages.

McKenzie and Terrill (1) and Grant (4) have reported that the secretion tended to be thick and tenacious during dioestrum and to become thinner at proestrum or during estrus.

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Cole and Miller (5) have made the only report concerning the changes in the height of the cervical epithelium. They reported that the superficial layer of cells is somewhat reduced in height during late metestrum and diestrum.

Reports of the investigators of cyclic changes in the uterine glands and mucosa agree fairly closely. In general, increased growth and coiling of the uterine glands during metestrum are reported. McKenzie and Terrill (1) also reported that both the surface and glandular epithelium were highest during the luteal phase.

Experiments showing the effects of estrogens have been relatively numerous in laboratory animals. Repair of atrophic changes brought about by castration is made as a result of injections of estrogens. Typical estrus symptoms and changes in the genital tract are also brought about by injections of estrogens into ovariectomized laboratory animals.

Corner and Allen (6) were the first to prove conclusively, by means of extracts, that progestational changes in the rabbit endometrium were due to a specific corpus luteum hormone. Hisaw and Leonard (7), in addition to confirming the results of Corner and Allen, found that the uterus must first react to estrogen before the corpus luteum extracts can produce progestational changes. Allen (8), however, has reported that progestational proliferation may be obtained in castrate rabbits by the use of progestin without preliminary treatment with estrogen. These results were secured, however, only in occasional animals.

Hisaw, Meyer and Fevold (9) have shown that premenstrual conditions can be produced in the endometrium of castrate monkeys by injections of theelin followed by injections of corporin. They reported, as evidence of corpus luteum activity, extensive coiling and dilation of the glands, increases in size and secretory activity of the cells in the glandular and surface mucous membranes, and almost complete absence of mitotic activity.

EXPERIMENTAL PROCEDURE

In this study 30 yearling ewes were spayed, allowed to recover from the operation, and were treated to demonstrate the effects of estrogen and progestin upon the sexual activity and the histology of the reproductive tract. Estradiol benzoate,[3] and progesterone synthesized from cholesterol by the method of Spielman and Meyer[4] (10) were used. Both materials were injected subcutaneously and in oil solutions.

Four of the spayed ewes received no injections; 5 received estradiol and were killed 12 days after injections; 4 others received estradiol alone but were killed 2 to 3 days after injections while showing signs of heat; and 14 ewes received estradiol, following which they were divided into three groups and given different doses of progesterone.

RESULTS

Occurrence of Estrus (Receptivity to a Teaser Ram)

All but one of the 22 ewes given 1000 R.U. of estradiol came into heat (table 1). The length of the induced estrual periods could not be determined in those ewes which were killed in heat. The average length of heat periods in the other groups were as follows: estrogen-12 day, 2.8 days; estrogen-high progesterone, 3.4 days; estrogen-medium progesterone, 3 days; and estrogen-low progesterone, 4.5 days. Nine of the treated ewes came into heat the day following the injection of estradiol, while the remaining 12 ewes were in heat by the second day following the injection.

Vaginal Smears

Vaginal smear records were taken daily and a comparison made of ewes given estradiol alone, estradiol plus the higher doses of progesterone, and of control ewes over a similar period of 13 days (fig. 1).

[3] Progynon-B, Schering Corporation.

[4] Grateful acknowledgment is made to Dr. M. A. Spielman Department of Chemistry for the preparation of the progesterone and to Dr. R. K. Meyer, Department for Zoology for its assay. Guinea pigs were used as assay animals, using the technique presented by Hertz, Meyer, and Spielman (11). The minimum amount of progesterone required to produce the copulatory reflex was taken as one guinea pig unit. A rabbit assay, according to the method of Corner and Allen (6), was also made. (One RB.U. = 20 G.P.U.)

TABLE 1. TREATMENT AND OCCURRENCE OF HEAT OF SPAYED EWES

No. of ewes	Treatment	Length of heat period, days		No. of days from estradiol injection until appearance of heat	
		Average	Range	Average	Range
4	'Controls'—No treatment	None	—	—	
5	'Estrogen-12 day'—1000 R.U. estradiol and killed 12 days later	2.8	0–3	1.8	1–2
4	'Estrogen-heat'—1000 R.U. estradiol and killed in heat (2–3 days after injection)	Impossible to determine		1.3	1–2
5	'High progesterone'—1000 R.U. estradiol followed, beginning on the fourth day, by 8 daily injections of 130 G.P.U. progesterone. (Killed 12 days after estradiol injection)	3.4	2–5	1.4	1–2
4	'Medium progesterone'—Same as 'High progesterone' group except the 8 daily injections were of 64 G.P.U. of progesterone.	3	2–4	2	2
4	'Low progesterone'—Same as the above progesterone treated groups except the 8 daily injections were 32 G.P.U. of progesterone.	4.5	3–6	1.5	1–2

The consistency of the mucus varied little in the control group and was usually rated as 'thick.' The consistency of the mucus of the ewes in both the estradiol-treated and estradiol plus high progesterone treated groups were very similar. The injection of estradiol tended to cause a thinning of the mucus on the day after injection, followed by a thickening on the second to third day. The thickness generally persisted for several days. Commonly by the 10th day after the first injection the mucus was of the consistency preceding the injections.

The numbers of 'scales' and epithelial cells in the smears of untreated spayed ewes, although showing considerable variation from day to day, showed no regular trends or peaks. Variations of these cell types in the smears of the two treated groups were very similar. The greatest number of 'scales' and 'epithelial' cells in the ewes of both groups commonly reached a peak about 5 or 6 days after the injection of estradiol. There appeared no outstanding differences in the occurrence of the leucocytes in the three groups.

Histological Studies

The reproductive organs of the injected and control ewes were removed immediately after slaughter. Two samples were taken from the oviducts, one approximately an inch from the ovarian end and another near the beginning of the uterine horn. One sample of an uterine horn was taken next to the body of the uterus, and another was obtained approximately an inch from the beginning of the uterine tubes. One sample was taken of the mid-region of the cervix, and two were taken from the vagina. One of the vaginal sections was taken near the external os of the cervix and the other anterior to and near the urinary orifice.

Bouin's and Helly's solutions were used as routine fixatives. This material was sectioned and a minimum of 10 sections, at least 100 micra apart, were taken from

TABLE 2. MEASUREMENTS OF THE GENITAL TRACT. UNTREATED AND AFTER TREATMENT WITH ESTROGEN OR PROGESTERONE

Treatment	No. of ewes	Statistics in micra or No.	Height of Epithelium											Coiling of Uterine Glands[4]			
			Vagina				Uterus						Cervix	Mid-mucosa		Base mucosa	
			Between papillae[1]		Above papillae[1]		Cotyledonary area[3]		Inter-cotyledonary area[3]		Glands[3]						
			Anterior region	Posterior region	Anterior region	Posterior region	Upper region of horn	Lower region of horn	Upper region of horn	Lower region of horn	Upper region of horn	Lower region of horn		Upper region of horn	Lower region of horn	Upper region of horn	Lower region of horn
Control	4	Mean	134	124	70	62	16	17	14	16	10	13	12	64	52	128	115
		Sum of squares[2]	7,033	24,555	3,212	7,232	593	588	2,686	3,815	5,878	4,856	268	30,991	18,335	22,752	49,939
Estrogen 12 day	5	Mean	149	143	77	78	18	17	16	17	13	15	18	43	35	129	109
		Sum of squares	22,306	10,438	4,135	764	3,473	175	1,494	629	1,157	471	2,012	1,357	1,277	21,007	2,635
Estrogen Heat	4	Mean	173	152	88	86	31	32	25	29	19	21	25	28	29	75	94
		Sum of squares	5,715	33,098	4,234	1,741	7,353	7,923	1,853	8,305	700	1,214	300	4,892	859	23,678	48,103
High Progesterone	5	Mean	165	157	83	75	32	36	25	31	19	20	25	104	75	203	152
		Sum of squares	4,643	9,011	6,837	1,208	11,346	11,802	11,707	7,220	774	581	701	19,847	8,240	24,964	23,332
Medium Progesterone	4	Mean	154	162	80	81	24	27	20	24	17	18	21	87	74	163	138
		Sum of squares	3,110	4,295	2,997	6,978	3,214	359	5,747	695	483	93	1,422	27,647	9,062	32,456	12,110
Low Progesterone	4	Mean	163	147	84	71	24	23	22	23	16	18	19	69	68	150	131
		Sum of squares	5,461	21,881	684	3,345	4,121	10,121	3,428	4,473	186	512	1,779	32,107	11,500	18,944	6,529

[1] Ten different measurements on each ewe. [2] Sum of squares between ewes within each treatment. [3] One hundred measurements on each ewe. [4] Measured by number of glandular cross-sections per field.

For comparing treatments, the following formula should be used for calculating "t." (Refer to Fisher's tables for use of "t"):

$$t = \frac{\text{Mean difference}}{\sqrt{\frac{\sum d_1^2 + \sum d_2^2}{n_1 + n_2 - 2}\left(\frac{1}{N_1} + \frac{1}{N_2}\right)}}$$

$\sum d^2$ = sum of squares for respective treatments
n = number of ewes for respective treatments
N = total number of observations on all ewes within the treatment

each sample. Mayer's hemalum and Orange G, and Heidenhain's iron hematoxylin and Light Green were used to stain the material. Muci-carmine was used as a mucin stain.

Vagina. Measurements of the height of the epithelium were made both between the stromal papillae and above the papillae (table 2). A great deal of variation was found in the height of the epithelium between ewes within the variously treated groups. Although average heights for the different groups appeared to show some effects of treatment, differences were significant in only a few cases. Consistent real differences in height of the epithelium were shown only between the control group and the group receiving estradiol in which the ewes were killed while in heat.

Cornified layers were seen in the vaginal sections from all ewes regardless of treatments. There was a slight tendency, however, for more cornification to be present

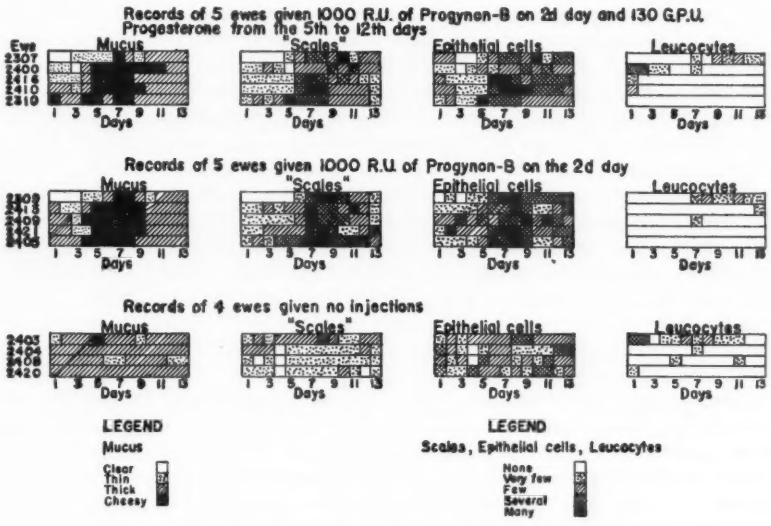

Fig. 1. Vaginal Smear Records of Injected Castrate Ewes.

in the vaginal sections of those ewes killed in heat. Leucocytes were also found in all of the sections, but appeared to be less numerous in the ewes killed in heat and more prevalent in those ewes which received both estradiol and progesterone. Estimation of their relative numbers in the ewes of the variously treated groups gave the following average results: control and estrogen-12 day, very few to few; estrogen-heat, very few; and progesterone groups, few to several.

Uterus. Measurements were made in sections taken from the upper and lower portions of the uterine horns. The height of the surface epithelium of the uterus in both the cotyledonary areas and intercotyledonary areas, and the height of the epithelium lining and uterine glands are recorded separately for each region (table 2).

In every instance comparisons of the height of epithelium showed a highly significant difference between the two estradiol-treated groups whether in the cotyledonary areas, inter-cotyledonary areas, or glands and in both the upper and lower portions of the uterine horns. The epithelium of the ewes killed in heat was consistently higher than that of ewes given the same amount of estradiol but killed 12 days after the injections. There were no significant differences in any case between the estradiol-

treated group of ewes which was killed in heat and the group which received estradiol and the high level of progesterone. This latter group had significantly higher glandular and surface epithelium than the estradiol-treated ewes which were killed 12 days after the injections. The epithelium of the ewes receiving the lower doses of progesterone was likewise higher than that of the ewes of this estradiol-treated group.

Only occasional highly significant differences were found between the height of the epithelium in the groups of ewes receiving the three different levels of progesterone. There was a decided trend, however, toward lower epithelium in the groups receiving the lower levels of progesterone.

No significant differences were shown in the uterine epithelium between the control group and the estradiol-treated group in which the ewes were killed 12 days after injections. There was a tendency, however, toward a greater epithelial height in the glands of the treated ewes.

In an effort to determine whether the uterine glands showed a difference in the amount of coiling between the variously treated groups, counts were made of the number of cross-sections of the glands appearing in a microscopic field (×352). It was assumed that an increase in the number of cross-sections indicated an increase in the amount of coiling. Counts were made in similar areas in glandular regions midway between the muscular layer and the lumen, and also in areas adjoining the muscular coat (table 2).

In nearly every case the groups given estradiol, plus one of the two higher levels of progesterone, gave a significantly higher number of cross-sections of uterine glands than either of the two estradiol-treated groups. There was a tendency toward somewhat more coiling in the estradiol-treated group killed 12 days after injections than in the group given the same injections, but killed in heat. Differences in gland counts between the ewes receiving the different levels of progesterone were seldom significant, nor were there uniformly significant differences between the progesterone treated animals and the control ewes.

Leucocytes and mitotic figures were observed in the uterine sections from all ewes, but no counts or estimations of numbers were made. No striking differences were apparent between the variously treated ewes. The nuclei of the epithelial layers tended to be more basal in the estradiol-progesterone treated groups than in the other groups.

Cervix. Study of the cervix was confined primarily to the comparative height of the cervical epithelium and the amount and characteristics of the cervical mucus.

All groups of ewes receiving injections had significantly higher epithelial layers than the control animals. The epithelium of the ewes killed in heat and that of the ewes receiving the highest level of progesterone were very similar. Both were significantly higher than the epithelium of the estradiol-treated individuals which were killed 12 days after the injections. This latter group and the ewes receiving the lower levels of progesterone showed no real difference in the height of their epithelium.

All of the cervical sections were stained with muci-carmine to demonstrate the presence of mucus. Virtually no secretory activity could be detected in the ewes of the control group. The amount of mucus in the sections from the ewes killed 12 days after the injections of estradiol was variable, but tended to be rather scant and dark staining in most sections. The sections from those ewes killed in heat showed more mucus, both in the cells and in the cervical lumen. The secretion stained a very light pink and that found in the lumen appeared to be thin and homogeneous. The greatest amount of mucus was shown in those ewes receiving estradiol and the high level of progesterone. The secretion was especially evident at the margins of the epithelial cells and also in the lumen. The material took a very bright red stain as contrasted to

the lighter staining mucus of those ewes killed in heat and also appeared to be thicker and more stringy.

Mucus was variable in amount and staining reaction in the sections from the ewes receiving the lower levels of progesterone, but generally was similar to that found in the estradiol-treated ewes which were killed 12 days after injections.

DISCUSSION

Heat was induced by injections of estradiol with possibly greater regularity in the spayed ewes than in the unspayed animals. Twenty-one out of 22 spayed ewes came into heat following injections of 1000 R.U. of estradiol. Previous work had resulted in only 5 of the 8 unspayed ewes which were injected with estradiol coming into heat. The spayed ewes were injected later in the season than were the unspayed, and this fact may have had a decided influence upon the results obtained. The most of the spayed ewes were injected during October—that is, during a month in which ewes would normally be expected to show signs of estrus. The larger proportion of the unspayed ewes, however, were injected during May, June, and July—normally months of anestrum. The slight increase in age of the spayed ewes may likewise have been a factor influencing the production of heat.

The teaser rams were more active during October than they were during the hot summer months, and ewes coming into heat may possibly have been overlooked during the earlier periods.

No reports of vaginal smears taken from immature or spayed ewes are found in the literature. The changes in the smear during artificially induced estrus or ovulation in the unspayed ewes or during estrus in the spayed ewes are in general similar to those described in normal cycling ewes. No marked differences were seen between the smears of ewes which ovulated without showing signs of estrus and those smears of ewes which came into heat but did not ovulate. It would be impossible with the technique used in this study to determine, by means of the vaginal smear, whether a ewe showing signs of heat had ovulated or not. Likewise, the smears of spayed ewes treated with estradiol alone were very similar to those of ewes given estradiol and progesterone. It would appear that the changes in the vaginal smear are produced primarily by estrogens and influenced little, if at all, by progesterone.

The smears of most of the ewes continued to show varying amounts of the various cell types after being spayed. In some instances smears similar to those seen at estrus were found 2 months after the spaying operation. There was no apparent correlation between the smears and the injections made previous to spaying. The ovaries were cleanly removed in all but 2 ewes, and these individuals were eliminated from the study. If estrogens are solely responsible for changes in the vaginal smear, the question naturally arises regarding their source in those spayed ewes showing considerable vaginal activity.

The histological study of the vaginal sections from the ewes in the variously treated groups showed significant differences in epithelial height only between non-injected ewes and the estradiol-treated group in which the ewes were killed in heat. There was, however, a distinct tendency for the height of the epithelium of the ewes killed in estrus to be greater than in the ewes of any other group. Previous studies of the changes in the vagina of the ewe during the estrual cycle have shown the epithelium to be highest during estrus.

There was a tendency for the leucocytes to be found in greater numbers in the vaginal sections from those ewes given estradiol and progesterone. This appears to be in harmony with the previous reports that leucocytes are most numerous in the vagina of the cycling ewe during the period of greatest activity of the corpus luteum.

The possibility of progesterone playing a role in increasing the number of leucocytes cannot be shown definitely in this study. No such role is indicated from a comparison of the vaginal smear records of ewes receiving estradiol and those of ewes receiving estradiol and progesterone (fig. 1).

The studies of the uterine sections demonstrate clearly the effect of estradiol in producing an increased growth of the glandular and surface epithelium. The uterine epithelium of those ewes killed in heat was commonly twice as high as that in the ewes of the control group. The rapid regression of this increased height was shown in those ewes given estradiol and killed 12 days after the injections. In this group the height of the epithelium averaged no higher than that of the control ewes.

The effect of progesterone in maintaining the increased epithelial height was clearly shown in the group of ewes receiving estradiol and the high level of progesterone. These also were killed 12 days after the injection of the estradiol, yet their uterine epithelium was just as high or slightly higher than that of the ewes killed in estrus. McKenzie and Terrill (1) have reported that both the glandular and surface epithelium of the uterus of the ewes studied were highest during the luteal phase of estrual cycle. If the effect of progesterone upon the uterine epithelium is merely to maintain the height already produced by estrogen, the greater height found during the luteal phase by McKenzie and Terrill could not be explained by an increased progesterone alone. It is possible that continued estrogen release from the ovary after ovulation causes the uterine epithelium to become higher than at estrus, and secretion from the developing corpus luteum may tend to maintain this increase in height. It must be recognized, however, that the relationship between the amounts of estrogen and progesterone at different stages of the estrual cycle in normal ewes may be quite different from that attained in the experimental ewes in this study.

Progesterone in combination with estradiol was shown to increase the amount of uterine gland coiling in the ewe. An increased amount of coiling has been reported during metestrum in the normal cycling ewe by Casida and McKenzie (2), Cole and Miller (5), and McKenzie and Terrill (1). This period corresponds in general to the period of greater size of the corpus luteum. No difference in the size of the glands measured by the height of their epithelium could be seen between the ewes killed in heat and the progesterone treated groups. The glands were much straighter, however, in the ewes which were killed in heat.

There were only occasional significant differences between the amount of gland coiling per unit area in the control group and the high progesterone treated ewes. However, comparative measurements of uterine cross-sections (made from camera lucida drawings) indicated that the uteri of untreated spayed ewes were only about one-half as large as those from injected ewes. The total number of cross-sections in the high progesterone treated ewes was, therefore, about twice as great as that of the control ewes. Estrogen very clearly causes an increase in total cross-sectional area of the uterus and the size of the glands. The latter, however, are apparently straightened out in the increased area of the endometrium.

The study of the cervical epithelium gave results somewhat similar to that of uterine epithelial layers. The greatest height was found in those ewes killed while in heat and the high progesterone treated group. The regression of the cervical epithelium following estradiol treatment was not as rapid as in the case of the uterine epithelium. The cervical epithelium of the ewes killed 12 days after receiving estradiol was still significantly higher than that of the control ewes. Progesterone, when given at the high level, maintained the height of the epithelium as it did in the case of the uterine epithelium.

If Cole and Miller's (5) observation that there is a slight decrease in the height

of the superficial layer of cells in the cervix during late metestrum and diestrum is generally true, then the highest level of progesterone effective in these experiments may have been slightly greater than that effective during the luteal phase of the normal cycling ewe.

Progesterone given at the highest level caused an apparent thickening of the mucus of the cervix. Mucus was much greater in amount in the cervical lumen of the ewes of this group than in ewes of any other group. Whether the increased thickness caused more to remain in the sections or whether there was an actual increased amount could not be definitely determined by this study.

There was little difference in the amount of mucus within the cells of the estradiol-treated ewes which were killed in heat and the high progesterone treated ewes. The mucus stained much darker, however, in the ewes of the latter group and the epithelial cell margins of these ewes were lined by strings of dark staining mucus.

Studies of the cervix have generally shown a greater amount of secretory activity during the late luteal phase and proestrual portion of the cycle. McKenzie and Terrill (1) and Grant (4) have reported that the secretion tended to be thick and tenacious during diestrum and to become thinner at proestrum or during estrus. These findings, together with the effects seen in this study, indicate that progesterone causes a thickening of the mucus and estrogen produces a thinning effect.

SUMMARY

Vaginal smear records taken on the ewes included in this study showed no differences between the smears of spayed ewes given estradiol alone and estradiol followed by progesterone.

Injections of estradiol resulted in an increased height of vaginal epithelium of spayed ewes. The addition of progesterone caused no apparent change in the histology of the vaginal sections from that produced by estradiol alone.

Estradiol also caused an increase in the height of the epithelium of the uterine glands and of the uterine surface of spayed ewes. This effect was rapidly lost. Within 12 days after the injections, the epithelial height was near that of the uninjected castrates. Injections of progesterone, following estradiol treatment, tended to maintain the increased height of the epithelium.

Uterine glands were found to show little coiling in those spayed ewes given estradiol and killed in heat. The addition of progesterone, even at the lower levels, caused an increase in the amount of gland coiling.

Injections of estradiol greatly increased the height of the cervical epithelium, and progesterone appeared to maintain the increased height. Regression was relatively slow. An increased amount of thicker, more darkly staining mucus was seen in the cervical sections of those spayed ewes given estradiol plus the high level of progesterone.

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URINARY ELIMINATION OF ESTROGENS INJECTED IN DOGS

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ARNOLD, HOLTZ AND MARX stated in 1936 that dogs treated with large doses of estrogens, estradiol benzoate, in daily doses of 5 mg. of the crystalline substance showed a severe anemia and died from the effects (1). This observation has been confirmed by other investigators (2, 3, 4). Simultaneous administration of androgens (androsterone) and estrogens did not prevent the latter from exerting its toxic action on the animals (3). It has not yet been determined why dogs should be more sensitive to estrogens than other animals including man. Since the metabolism or elimination mechanism in the dog may differ from that of other animals, it appeared pertinent to analyze the elimination of administered estrogens in the urine and their distribution in various organs.

METHODS

The hormones used were dissolved in olive oil, 1 cc. containing 5 mg. of the crystalline substance. Injections were given subcutaneously. The amounts of estrogens were determined from time to time in the urine and occasionally also in the blood. After death, the liver, kidneys, heart, lungs and brain, were also examined. Both forms, i.e. free and combined hormone, were always determined on every sample. The method of extraction of the urine used was that described by one of us (5, 6) for the determination of comb growth promoting substances in the urine. The extracts were assayed for their content of estrogens on castrated mice, for androgens on the capon comb. Organs were chopped finely and mixed with 3.5 vol. of 96% alcohol. This mixture was allowed to stand overnight and filtered on a Büchner funnel the following morning. The residue was again treated with 70% alcohol and filtered. The combined alcoholic solutions were evaporated in vacuo and the aqueous residue was then treated like urine.

Determinations were made on the urinary estrogens in dogs after administration of *a*) estrone and *b*) a mixture of estradiol benzoate and testosterone. The latter experiments were carried out to determine whether the simultaneous administration of testosterone might counteract the toxic effect of the estrogens. Since it has been suggested that estrone and estradiol are converted under the influence of the uterus into less active substances (7), some of the experiments with estradiol benzoate were carried out on hysterectomized dogs. A study was also made of the androgen and estrogen excretion in the urine and the androgen content of various organs in animals treated with testosterone alone and after the administration of diethylstilbestrol.

Administration of Estrogens

Estrone, in the daily dose of 5 mg. (50,000 I.U.) was injected in *dog 28*, a female, and *26*, a male. The results of the urine assays are given in table 1. Partly in the free and

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partly in the combined state, 7.2% of the estrogenic activity injected during 5 days in *dog* 28 and 12 to 16.5% of the estrogen injected during 18 days in *dog* 26 was recovered in the urine.

Estradiol benzoate, in the dose of 5 mg., equivalent to 40,000 I.U., and simultaneously testosterone, 10 mg. and 5 mg., respectively, were injected daily into the

TABLE I

Dog No., sex	Estrogen injected daily subcutaneously	Urine collected	Estrogens in the urine				Estrogenic activity in urine	Remarks
			Free	Ratio keto: non-keto	Combined	Ratio keto: non-keto		
			I.U.		I.U.		%	
			A. Normal Dogs					
28♀	Estrone, 5 mg.	7/23–28	5800	1:4	8700	1:5	7.2	Total amount injected, 7/23–7/28: 20 mg. of estrone (200.000 I.U.)
26♂	Estrone, 5 mg.	12/5–7 12/14–18 12/19–22	8000 16000 15000	2:1 7:6 10:7	10000 20000 18000	1:4 1:2 2:3	12.0 14.4 16.5	For 19 days (12/5–12/23) daily injected. Total amount of estrone 95 mg.
19♂	Estradiol benzoate, 5 mg. (10 mg. respect)+ testosterone 10 mg.	7/1–7 7/8–13 7/24	20000 20000 4000	2:5 — 2:3	5000 5000 500	1:4 — 1:5	9.0 10.5 11.0	Received 5 mg. of estradiol benzoate, 6/26–7/24, and 10 mg. 7/25–8/1, daily. Daily dose of testosterone 10 mg.
23♂	Estradiol benzoate, 5 mg.+ testosterone 5 mg.	10/18–20 10/21 10/22–24 10/25–27	25000 10000 25000 25000	1:2 — — —	25000 10000 5000 10000	1:5 — — —	40.0 50.0 25.0 30.0	Injected daily for 20 days, 10/11–10/30.
			B. Hysterectomized Dogs					
4♀	Estradiol benzoate, 5 mg.	4/9–18 4/19–29	40000 60000	— 1:1	20000 20000	1:4 —	15.0 20.0	Injected daily from 3/16. Died on the 44th day after 44 injections.
5♀	Estradiol benzoate, 5 mg.	4/9–13	15000	1:1	10000	2:1	12.5	Injected daily from 3/16. Died on the 32nd day after 31 injections.

male dogs 19 and 23. The recovery in the urine of *dog* 19 was 9 to 11% of the amount of estrogen given over a period of 28 days and 25 to 50% of the amount administered during 20 days in *dog* 23. The illness was much more acute in the latter animal, the urine of which showed the highest estrogenic activity among all injected dogs. The dog died after treatment for only 22 days.

Dogs 4 and 5, from which the uterus and vagina were removed, were subsequently daily treated with 5 mg. of estradiol benzoate. Twelve and five-tenths per cent of the estrogen administered during 5 days in *dog* 5 and 15 to 20% of the estrogen injected during 20 days in *dog* 4 could be demonstrated in the urine. Comparing the values given in table 1 for free and combined hormone in the urine, one notices the high content of free, lipoid-soluble hormone after administration of estradiol benzoate.

From the publication of Doisy and his collaborators (8) we know that after administration of the ketone compound (estrone) to monkeys, non-ketonic estrogenic substances can be detected in the urine. Conversely, after administration of the non-ketonic compound (estradiol), an estrogenically active ketone compound was detected in the urine.

Also in dogs, as it is apparent from table 1, estrogenic non-ketonic and ketonic compounds can be detected in the urine after administration of estrone and estradiol respectively. The ratio of ketonic to non-ketonic compounds, especially in the combined form is considerably in favor of the non-ketonic fraction, no matter whether the ketonic compound (estrone) or the non-ketonic compound (estradiol) is given.

Estrogens are eliminated in human urine chiefly in two forms, namely, as estrone and as estriol; estradiol is eliminated in smaller amounts. Marrian (10) was the first to isolate estriol in considerable quantities from pregnancy urine. This discovery has been repeatedly confirmed by other investigators. On the basis of differences in the results between the biological and the colorimetric standardization of the non-ketonic fraction Smith, Smith and Pincus (11) have expressed the opinion that estradiol must also be eliminated in pregnancy urine along with estriol. This has been proved by the isolation of estradiol from pregnancy urine by Doisy et al. and by David (12).

Doisy and others (13) have shown from an examination of pig ovaries and the follicle liquid derived from them that the hormone produced by the ovaries consists chiefly of estradiol contaminated with a small percentage of estrone. Dingemanse and Mühlbock (14) found 90 to 95% of the estrogenic activity of the liquid from horse follicles to be due to non-ketonic and 5 to 10% to ketonic compounds. Thus it may be that in some species the estradiol produced in the ovaries is converted into estrone and the much less active estriol.

By means of the David reaction (15) Pincus and others (7) detected large amounts of estriol (but no estradiol) in the urine of normal rabbits after treatment with estrone or estradiol. These results may indicate that the estrogenic activity, which we found in the non-ketonic fraction, is caused chiefly by estriol, and the activity in the fraction containing the ketonic compounds, by estrone.

In order to calculate how much of the estrone and estradiol administered eventually appears in the urine, it is necessary to know the physiological activity of estradiol, estrone and estriol. The international unit is based on the estrogenic action of estrone, 1 I.U. being the activity produced by 0.1γ of estrone. The estrogenic activity of estradiol and its benzoate is also accurately known. These compounds are 2 and 0.8 times as active as estrone, respectively, when tested in mice. The estrogenic activity of estriol has been determined less accurately. We, ourselves, found the activity of estriol to be only 1/10 that of estrone as tested in castrated female mice, i.e., the unit of estrogenic activity is present in 1γ of estriol. With this in mind it is possible to calculate how much of the estrogenic substance administered eventually appears in the urine, supposing the whole non-ketonic fraction consists of estriol. The first urine from *dog 26* will be used as an example. This urine was found to contain 8,000 I.U. in the free state. The ratio of estrone to estriol is 2:1, thus 5,333 I.U. are in the form of estrone and 2,666 I.U. in the form of estriol. Ten thousand I.U. were eliminated in the combined form, of which 2,000 I.U. were in the form of estrone and 8,000 as estriol. Expressed in weight units this represents 0.5333+0.2 mg. = 0.7333 mg. of estrone and 2.666+8.0 mg. = 10.666 mg. of estriol. Thus the total amount of estrogenic material eliminated was 11.4 mg. During the time 3×5 mg. = 15. mg. of estrone was administered. Thus, the total recovered in the urine would be 76%. However, the possibility that some of the non-ketonic estrogen excreted was estra-

diol must be considered. Were this the case the percentage of the recovery would be much less than 76%.

Reference should also be made to the results obtained in hysterectomized dogs. According to Pincus (7) estriol is formed in the organism only in the presence of the uterus. However, the ratio between ketonic and non-ketonic compounds in the urine of the hysterectomized animals did not differ very much from the ratio found in normal female dogs.

The content of estrogenic substances in the organs of various dogs was determined as well as that in the urine. A summary of the observed values is given in table 2. The content of estrogens in the various organs of the same animal and that in the

TABLE 2

Dog No., sex	Duration of treatment	Total amt. injected	Estrogens found in the organs before and after hydrolysis										Remarks
			Liver		Kidneys		Heart+lungs		Brain		Spleen		
			Before	After	Before	After	Before	After	Before	After	Before	After	
			I.U.	I.U.	I.U.	I.U.	I.U.	I.U.	I.U.	I.U.	I.U.	I.U	
						A. Normal Dogs							
2♀	1/19–2/13	Estradiol benzoate, 125 mg. (1,000.000 I.U.)	600	900	100	150	—	—	—	—	—	—	Died on 2/14. Weight of the liver 308 gm., spleen 20 gm., kidneys 70 gm.
8♀	3/16–4/11	Estradiol benzoate, 130 mg.= 1,040.000 I.U.	90	3000	40	50	heart <60	heart <60	40	<30	<30	<30	Died on 4/11. Weight of the liver 320 gm., spleen 30 gm., kidneys 64 gm.
11♀	3/20–4/11	Estradiol benzoate, 100 mg.= 800.000 I.U.	100	1000	<30	<30	heart <60	heart <60	—	—	—	—	Died on 4/11. Weight of the liver 450 gm., kidneys 64 gm.
9♀	3/24–4/13 6/26–7/28	Estradiol benzoate, 100 mg. Estradiol benzoate, 160 mg. Total: 260 mg. =2,080.000 I.U.	800	800	100	75	<30	300	120	50	<30	300	Animal killed on 7/28. Weight of the liver 300 gm., kidneys 62 gm., spleen 20 gm., brain 200 gm., heart+lungs 215 gm.
						B. Hyster- and Ovariectomized Dog							
3♀	1/19–2/15	Estradiol benzoate, 135 mg.= 1,080.000 I.U.	300	4000	80	700	—	—	—	—	—	—	Died on 2/15.

same organ from different dogs varied considerably. This may be due to the fact that the animals did not die or were not killed at the same time after receiving the last injection. In relation to its weight, the liver was always richer in estrogens, than the other organs. The highest content amounted to 10% of the dose given the previous day.

Administration of Androgens

In two male dogs a study was made of the urinary output of androgens and estrogens and the androgen content in the organs after injection of testosterone and testosterone propionate, respectively. One of the animals received in this way 5 mg. of testosterone daily for 63 days and then 10 mg. daily for 17 days more, while the other animal was injected for 81 days with 10 mg. of testosterone propionate daily, making a total of 810 mg. Right up to the end of the experiment neither of the animals showed any signs of illness, nor could any abnormalities be observed at the autopsy. Thus, unlike estrogens, androgens had no toxic properties.

During the experiment, the urine from 5 days was collected and then examined. In no single case could 1.5% of the amount of activity administered be detected by

injection into the comb of the capon. In this connection we should like to mention that a positive comb growth promoting action is never observed with the 24-hour urine of normal dogs, not even when the Fussgänger test is used.

From the above mentioned results it was not probable that the organs of these animals would show strong androgenic action on the comb. In fact no comb growth promoting activity could be detected even by the Fussgänger test, with extracts of the liver, kidney, spleen, heart or lungs. Extracts of feces collected over a period of 24 hours showed no androgenic activity. Neither could any androgenic activity be found by means of the Fussgänger test in 70 cc. of blood half an hour after injection of 10 mg. of testosterone propionate.

The determination of the urinary estrogens was suggested by the investigation of Steinach et al. (9), who found an increase in estrogens in the urine and in the blood of rats after implantation of testis tissue or after injection of testis extract. We cannot say with certainty that the estrogenic activity of the dog's urine is increased under the influence of testosterone. The highest daily elimination in the urine in one of the dogs occurred one month after the beginning of the treatment (urine collected over 11 days) and amounted to 17 I.U., while before treatment the 24-hour urine contained less than 10 I.U. Similar daily variations occur, however, in untreated animals. In the second dog, whose normal estrogen elimination was unknown, a daily urinary excretion of 50 to 100 I.U. was observed during the treatment, examined over one month. These values seem significantly high, but we have observed similar high values in untreated animals. If testosterone has any effect in this case on the elimination of estrogens, such an effect must be assumed to be very small.

Administration of Diethylstilbestrol

Three normal female dogs were treated with this substance in the same way as with estrone or estradiol benzoate, respectively. Besides the toxic properties, which were described in a previous communicaion, the urinary elimination of this substance (table 3) and its accumulation in the organs (table 4) was investigated. The urines were extracted in the same way as those containing estrone or estradiol benzoate, i.e. they were extracted both before and after hydrolysis. The small amount

TABLE 3

Dog No., sex	Urine collected	Estrogens in the urine		Estrogenic activity in urine	Remarks
		Free	Combined		
		U	U	%	
18♀	9/28–29	6000	1000	~ 9.0	Diethylstilbestrol, 5 mg. given daily for 41 days. Died on 10/28, weight 14.5 kg.
	9/30–10/1	6000	1500	~10.0	
	10/2	3000	750	~ 9.5	
	10/10–11	12000	2000	17.5	
	10/13–14	15000	3000	22.5	
	10/16–21	50000	10000	25.0	
	10/23–24	45000	500	57.0	
21♀	7/24	5000	300	13.0	Diethylstilbestrol 5 mg. given daily, 6/3–6/16, and then 6/27–7/27. Killed on 7/28, weight 10.9 kg.
	7/26	8000	1000	22.5	
22♀	10/7–8	10000	3000	~16.0	Diethylstilbestrol, 5 mg. given daily, 9/15–11/4. Died 4 days after the last (51st) injection. Weight 16.5 kg.
	10/10–11	12000	3000	~19.0	
	10/13–14	30000	3000	~41.0	
	10/16–17	10000	3000	~16.0	
	10/20–27	50000	4000	~17.0	

TABLE 4

Organs	Weight of organs			Estrogens found					
				Dog 18		*Dog 21*		*Dog 22*	
	Dog 18	*Dog 21*	*Dog 22*	Free	Combined	Free	Combined	Free	Combined
	gm.	gm.	gm.	U	U	U	U	U	U
Brain	63	70	—	25	25	25	25	—	—
Kidneys	2×50	2×34	—	25	50	25	25	—	—
Liver	—	440	460	—	—	1500	2000	50	800
Liver+spleen	550+60	—	—	300	200	—	—	—	—
Heart+lungs	110+160	—	—	25	25	—	—	—	—
Pancreas+suprarenal	—	34+1.2	—	—	—	50	50	—	—
Bile	29 cc.	—	27 cc.	60	600	—	—	100	800
Blood	—	—	25 cc.	—	—	—	—	10	500

of combined estrogenic substance (at most 33%) compared with the amount of free substance is noteworthy. One would have expected that a compound, which is no less toxic than the estrogens, estrone and estradiol benzoate, and which contains two hydroxyl groups, would have been rendered more or less harmless in the organism by combination either in the form of a glucuronide, a sulphate or a glycine ester.

In order to find out whether simple boiling of the neutral solution was sufficient to hydrolyze any ester eventually formed, we extracted part of the urine 6 times with ether at room temperature. This ether solution was also strongly active. After treating these urine extracts with Girard's reagent, the ketone fraction showed no estrogenic activity, which was to be expected. Only tertiary hydroxyl groups which do not give ketone groups on oxidation are present in diethylstilbestrol.

Table 4 shows that the liver had a considerably increased estrogenic activity as compared with other organs. The same also holds for the bile which contained 23 U per cc. in *dog 18*, and 33 U. per cc. in *dog 22*, for the greater part in the combined form. Also the estrogenic substances in the blood of *dog 22* were present entirely in the combined state. This is contrary to the condition in the case of urine wherein the estrogens are almost entirely in the free form.

The blood contained 20 I.U. of estrogens per 1 cc. This quantity was found in the blood two days after administration of the last 5 mg. of diethylstilbestrol.

After intravenous injection of estrone into dogs, Stamler (16) found that more than 90% of the estrogenic activity disappeared from the blood stream within a few minutes. About 13% of the estrogenic activity was recovered in the urine, not less than 13% was eliminated in the bile, while 1.26% was found in the liver, kidneys and spleen. After administration of estradiol benzoate the results were virtually the same as in our experiments, except that the ratio of free to combined hormone is changed more markedly in favor of the free hormone.

The amount of estrogenic substances eliminated after administration of diethylstilbestrol amounts to 50% of the quantity given and occurs mostly in the free state. The liver contains about the same amount of these substances as it does after administration of estrone or estradiol.

At present a question still to be answered is whether or not diethylstilbestrol exerts its physiological action in its original form. Evidence in favor of this action would be obtained if diethylstilbestrol could be detected in the urine. It is of course possible that diethylstilbestrol itself does not show a strong estrogenic action but affects the excretion of estradiol or estrone by the endocrine glands. Another possibility is that diethylstilbestrol is converted in the body into estradiol or estrone.

Since biological differentiation between estrone and estradiol on the one hand and diethylstilbestrol on the other is not easy, and the quantities present are as a rule so small, a specific color reaction for diethylstilbestrol would greatly facilitate the investigation. This color reaction can be obtained with antimony pentachloride. When a few drops of a 10% solution of antimony pentachloride in chloroform are added to a solution of diethylstilbestrol in the same solvent, a fuchsin red color is produced. In this way 1 to 2.5γ of diethylstilbestrol can be easily detected. We have succeeded in detecting 2.5γ, biologically standardized, by means of the above color reaction in the purified and decolorized urine of dogs which were treated with diethylstilbestrol. The estrogenic sterols do not give this color reaction; at most they produce a reddish brown coloration and then only in much higher concentration. Thus, it may be assumed that the increased estrogenic activity of the urine is due at least in part to stilbestrol. This method of determining the content of diethylstilbestrol in mixtures with estrone or estradiol, by means of antimony pentachloride, will be reported in a subsequent communication.

SUMMARY

In dogs treated daily with 5 mg. of estrone, 6.5 to 16% of the estrogenic activity appears in the urine. Supposing that the non-ketonic fraction would consist entirely of estriol, then 60 to 100% of the quantity of estrone administered could be recovered in the urine. A considerable part is eliminated in the combined form.

The amount of estrogenic substances eliminated in the urine of hysterectomized dogs after administration of estradiol benzoate amounts to 12.5 to 20% of the amount given. Also in this case a part appears in the ketonic fraction. If this is considered as estrone, and the non-ketonic fraction as estriol, 70 to 100% of the amount of material given is recovered in the urine. The greater part is excreted in the free form. Hysterectomized dogs treated daily with estradiol benzoate do not live longer than normal dogs undergoing the same treatment.

After simultaneous administration of testosterone propionate and estradiol benzoate into male dogs, a large proportion of the estrogenic substances in the urine are eliminated in the free state. Also in this case part was present in the ketonic form and part in the non-ketonic form. Nine to 11% and 25 to 50% respectively of the amount of units given were found again in the urine. Simultaneous administration of testosterone propionate does not suppress the toxic action caused by estradiol benzoate.

The quantities of estrogenic activity found in the organs after daily administration of estrone or estradiol benzoate are in general small as compared with the quantities administered. The highest concentration is observed in the liver. A considerable amount was present in the combined form, while part of the estrogens were encountered as keto compounds and another part as non-ketonic substances. Almost the same quantities and proportions were found in the organs of hysterectomized dogs after administration of estradiol benzoate.

Comb growth promoting substances could not be detected either in the urine, the feces, the blood, or in the organs after daily administration of 5 to 10 mg. of testosterone propionate over periods of 80 to 81 days. It is uncertain whether an increase in the amount of estrogenic substances occurs in the urine of dogs treated with testosterone; if there is any increase, it is a small one.

After administration of diethylstilbestrol, with two exceptions, 8 to 25% of the activity given was found in the urine, mostly in the free state. The liver showed the highest concentration of all organs. A considerable concentration was found in the bile, namely 23 and 33 U per cc., respectively.

The estrogenic activity of the blood was increased even two days after the subcutaneous injection of diethylstilbestrol, in contrast to the subcutaneous injection of estrone, in which case less than 2% of the activity given was found in the blood 45 minutes later.

After subcutaneous administration of diethylstilbestrol, this substance could be identified in the urine and in the liver by means of the antimony pentachloride reaction.

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BLOOD SERUM AND SKELETAL CHANGES IN TWO BREEDS OF DUCKS RECEIVING ESTROGENS[1]

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FLUCTUATIONS in the serum calcium level and in the degree of hyperossification of the long bones in the pigeon are correlated with ovarian function (1, 2). Similar changes also occur in sparrows in relation to the ovarian cycles (3), and follow the injection of estrogens in pigeons (4) and fowl (5, 6). Fowl, however, differ from pigeons in their response to estrogen in that the levels of serum calcium are much higher and hyperlipemia is grossly apparent. These differences in response might be associated with the fact that pigeons lay only 2 or 3 eggs in a clutch, whereas fowl lay more or less continuously. On this assumption, the amount of calcium and lipoid material mobilized under the influence of estrogen treatment would be to some extent proportional to egg production. With this in mind, the present investigation was begun on ducks since they not only afford an opportunity to test the effects of injected estrogen in another species but also permit the study within one species, of two breeds which vary in egg-laying potentialities.

The administration of extracts of the parathyroid glands has been reported to increase the level of serum calcium in fowl (7). Since the mechanism whereby estrogens affect the blood levels of calcium and mediate the formation of trabecular bone has not been investigated extensively it seemed advisable to determine whether or not the parathyroids are involved. Therefore, several ducks were treated with parathormone to compare their responses with the estrogen treated birds.

MATERIAL AND METHODS

Male Pekin and Mallard ducks were chosen for this work because they represent breeds of relatively high and low egg laying capacity, respectively. The Mallards were trapped in nature. In order to avoid seasonal fluctuations in serum calcium and lipids, such as have been associated with ovarian activity, only males were used. The first series, which was run from February 6 to February 27, consisted of 5 Pekins and 5 Mallards with 2 control and 3 treated animals in each group. The second series of experiments, conducted from March 21 to April 10, consisted of 3 control and 3 treated Pekins and 3 control and 4 treated Mallards. From 0.34 mg. to 2.0 mg. of estradiol benzoate[2] for the Mallards and 0.80 mg. to 2.0 mg. estradiol benzoate for the Pekins were administered intramuscularly in two injections daily over a period of 21 days (table 1).

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[2] The estradiol benzoate used in these experiments was generously supplied by the Schering Corporation through the courtesy of Dr. E. Schwenk.

TABLE 1. SERUM FAT, SERUM CALCIUM, SERUM ORGANIC PHOSPHORUS AND ENDOSTEAL BONE FORMATION IN DUCKS FOLLOWING ESTROGEN ADMINISTRATION

Number of animal	Treatment mg. daily	Serum calcium mg. %	Serum inorganic P_2 mg. %	Serum fat[1] mg. %	Endosteal bone formation[2]		
					Femur	Tibia	Tarsometatarsus
				Pekin Drakes			
First series							
2	control	10.40	2.4	448.5			
3	control	8.64	2.0	552.6			
1	0.80	23.52	5.6	642.1	+++++	++	o
4	1.04	52.40	9.9	3136.0	++++++++	+++(+)	(+)
5	2.00	68.00	9.0	6184.5	++++++++++	++++	(+)
Second series							
6	control	9.60	5.8	321.6			
7	control	9.60	4.0	247.4			
10	control	10.24	3.2	292.8			
8	1.04	32.00	9.0	830.4	+++++++	++++	o
9	1.04	36.80	8.0	589.8	+++++++	++++	o
11	2.00	54.56	10.6	1627.9	++++++++++	+++(+)	(+)
				Mallard Drakes			
First series							
1	control	10.40	4.8	—			
5	control	9.92	4.0	732.1			
2	0.34	30.40	6.1	—	+	(+)	o
3	0.52	23.84	5.6	855.6	++	+	(+)
4	1.00	27.33	—	2017.0	++(+)	+(+)	(+)
Second series							
6	control	—	—	—			
9	control	9.76	2.8	775.0			
11	control	9.76	3.8	760.4			
7	0.52	29.92	7.3	2876.0	++	+	o
8	0.52	22.40	5.4	1219.8	+(+)	+	o
10	1.04	46.80	14.0	8538.3	++(+)	+(+)	(+)
12	2.00	62.40	12.0	7672.3	+++	++	(+)

[1] The serum fat values are averages of 2 to 5 determinations.
[2] Graded by visual inspection, ten plus signs indicating complete obliteration of the marrow cavity.

Four Pekin drakes also received 5, 15, 30, and 50 units, respectively, of parathyroid extract[3] twice daily for 21 days. The ducks were kept in confined quarters and were permitted free access to water and a diet consisting of a balanced poultry mash and grain. The body weight of the estrogen treated Mallard drakes remained relatively constant throughout the experiment. However, some of the Pekins, both estrogen treated and control, showed a loss in weight (table 2).

The blood for chemical analysis was obtained by heart puncture just prior to autopsy. Serum calcium was determined according to Tisdal's modification of the Kramer-Tisdal method previously used (4), phosphorus according to the method of Fiske and Subbarow (8), phosphatase by King, Hazelwood and Delory's (9) modification of the method of King and Armstrong (10), and total serum and total blood lipids by the technic of Shaw (11). The animals usually died as a result of the blood loss; if not, they were decapitated. At autopsy the hypophyses, adrenals, thyroids, parathyroids, testes and livers were removed, dissected from the surrounding tissues and preserved for histological study. The bones of the legs, namely the femur, tibia, tarsometatarsus were dissected out and sawed longitudinally. The extent of new

[3] The parathyroid hormone was supplied by Dr. D. C. Hines of Eli Lilly & Company.

bone formation was determined by direct observation and rated in a relative scale (table 1).

RESULTS

The serum calcium levels were fairly uniform in the 9 untreated Pekin and Mallard drakes (table 1). They ranged from 8.6 mg.% to 10.4 mg.% in the Pekins (5 birds) and from 9.8 mg.% to 10.4 mg.% in the Mallards (4 birds). Follwing the injection of estrogen, the serum calcium increased markedly in both breeds of ducks. Values of from 22.4 mg.% to 68.0 mg.% calcium were obtained. The values tended

TABLE 2. BODY AND ORGAN WEIGHTS FOLLOWING ESTROGEN ADMINISTRATION

Number of animal	Body weight at beginning of experiment	Body weight at end of experiment	Testes	Liver
	gm.	gm.	gm.	gm.
		Pekin Drakes		
First series				
2	2820	2660	136.8	36.5
3	3230	3260	143.5	29.3
1	3340	2550	5.0	43.9
4	2830	2730	50.1	60.1
5	2520	2510	43.5	62.1
Second series				
6	3040	2550	179.1	40.7
7	2600	2380	178.9	30.8
10	3180	2730	215.8	34.2
8	2720	2750	127.0	62.2
9	2670	2610	188.6	42.2
11	2930	2620	177.3	45.0
		Mallard Drakes		
First series				
1	1170	1180	4.30	19.0
5	980	960	0.85	13.0
2	1080	1040	0.30	13.9
3	1120	1220	0.35	40.4
4	1220	1360	1.34	56.0
Second series				
6	990	1050	15.0	13.6
9	1290	1260	18.7	18.7
11	1200	1210	13.1	24.8
7	1210	1270	4.9	52.3
8	1100	1060	1.9	28.8
10	1020	1010	1.3	33.6
12	1160	1130	1.9	33.0

to be somewhat lower in the Mallards than in the Pekins. In the first series, where the estrogen was administered in amounts proportional to body weight regardless of breed, the smaller Mallards receiving approximately one-half as much estrogen as the Pekins, there was no close correlation in the Mallards between the amount of hormone administered and the level of serum calcium. One Mallard which received 0.34 mg. estradiol benzoate showed an exceptionally high serum calcium level. In both groups of Pekin drakes and in the second series of Mallards, the serum calcium levels increased uniformly with the dose regardless of the breed or the differences in body weight of the two breeds.

The extent of new bone formation differed greatly in the two breeds of ducks (table 1). The Mallard drakes, like the cock, exhibited only a limited amount of new

bone formation under the influence of relatively large dosages of estrogen, while the Pekin drakes showed extreme changes. In fact, in the latter breed, the marrow cavities of the femurs were almost completely obliterated in the birds which received the highest doses of hormone. The long bones of the untreated Pekin drakes, however, had a much greater amount of trabecular bone than did the Mallard drakes. This was probably a factor in bringing about the greater response of the Peking in the formation of new endosteal bone following the injection of estrogen. In both breeds of ducks, as in the cock, the amount of new bone formed in birds receiving estrogen decreased proximo-distadly from the femur to the tarsometatarsus, and in the latter the changes were very slight, even in the birds given the highest doses. Endosteal bone formation was the same in the two experimental series of Mallards and Pekins, respectively, when similar amounts of hormone were used. The extent of hyperossification showed a definite correlation with the serum calcium level in both the Mallards and Pekins. It is important also that in the Pekin drakes, extreme stages of new bone formation were obtained in mature animals and in the absence of growing cartilage. No evidence was found that calcification of soft tissues had occurred.

Associated with the serum calcium changes were increases in inorganic serum phosphorus. However, the phosphorus values were not as consistent in the control or the experimental animals, as the calcium values. The birds given estrogens all had higher serum phosphorus values than did the controls, but the increases were relatively less than for serum calcium (table 1). The phosphatase activity of the serum was increased greatly in the treated animals (12) as might be expected in association with the rapid proliferation of bone.

Estrogen treatment resulted in a marked rise in blood fat, similar to that found in fowl, but the two breeds of ducks showed definite differences in response. In considering the results obtained, however, it must be taken into account that untreated Pekin drakes had a much lower blood fat level than untreated Mallard drakes. The serum fat levels of the two control Pekins of the first series were 448.5 mg.% and 552.6 mg.% (table 1). In the second series, the serum fat levels of the 3 control Pekins ranged from 247.4 mg.% to 321.6 mg.%. On the other hand, the control Mallard drakes of both series showed serum fat values of from 732.1 mg.% to 775.0 mg.%. Apparently among the untreated Pekins, a definite lowering of the blood fat level occurred during the interval between the first and second series of experiments, while the blood fat levels of the Mallards either remained unchanged or increased slightly during the same period of time. Estrogen administration resulted in a sharp rise of serum fat in the first series of Pekins but a much less extreme increase in the second series of Pekins. In the Mallards, the reverse was true; the animals of the second series responded with a much more extreme increase in blood fat than did those of the first series. There was no obvious association between the increase of serum fat and that of serum calcium. The birds developing hyperlipemia showed marked hypertrophy (table 3) and discoloration of the liver. In the drakes receiving the larger doses of estrogen, the liver assumed a definite yellowish color. Histological examination revealed marked storage of fat in the liver, the extent of fat infiltration being proportional to the extent of hypertrophy and greater in the Mallards than in the Pekins. Acetone bodies in the blood serum increased in some of the estrogen treated birds.

The testes of the Pekin drakes were reduced in size, following estrogen administration in the first experimental series but not in the second (table 2). In the Mallards a great reduction in the testis weight occurred in the second series but not in the first (table 2). In both breeds of ducks the testes of the control birds enlarged during the period between Feburary 27 and April 10, the time between the two autopsy

dates. The adrenals and parathyroids of both control and estrogen treated ducks varied greatly in weight. Because of this variation it was impossible to draw any conclusion concerning the effect of the estrogen treatment. The largest parathyroids and adrenals occurred in some of the experimental birds but in other estrogen treated birds these glands were smaller than those of some of the controls. Microscopic examination of sections of these glands revealed no marked morphological changes that could be associated with the treatment. There was no consistent change in either hemoglobin content or red cell count of the blood.

Parathyroid extract, at least in doses up to 100 U daily did not produce comparable effects on serum calcium and serum lipids or on bone (table 3) to those following estrogen injection. Except for the animal which received the lowest dose, there was considerable loss in body weight. In the drake which received the highest dose the serum

TABLE 3. EFFECTS OF PARATHYROID EXTRACTS ON THE SERUM CALCIUM, SERUM FAT AND ENDOSTEAL BONE FORMATION IN THE PEKIN DRAKE

Number of animal	Units of extract daily	Body weight at start	Body weight at autopsy	Serum calcium	Serum fat	Bone changes
		gm.	gm.	mg. %		
1	10	2650	2600	11.9	138	0
2	30	2780	2300	11.9	245	0
3	60	2670	2180	11.9	369	0
4	100	2650	2290	12.8	219	0

calcium rose to only 12.8 mg.%. The serum lipid levels were low, varying between 138 mg.% and 369 mg.%. The bones did not show gross changes in internal structure.

DISCUSSION

From the results presented here, it may readily be seen that ducks repond to estrogen injection in the same manner as fowls (5), sparrows (13), and pigeons (4, 14), though quantitative variations exist between the species. These observations demonstrate that estrogenic hormones may be involved in some way in the mechanism controlling the metabolism of fats and calcium in birds. This is especially significant since associated with oviparity large amounts of concentrated nutrient are stored for the young and the egg is encased in a shell containing large amounts of calcium. In mammals these conditions do not exist, and serum calcium and lipid are not markedly increased (15). It seems that the influence of estrogens on fat and calcium metabolism in the bird is associated with egg laying and, therefore, the extent of the changes might be related to the egg laying capacity of the species of bird. As was pointed out in the introduction, the evidence from the species formerly studied suggested such a relationship. However, comparison between species is hardly justifiable. The limited material in the present experiment gives only an indication that the difference in the egg laying capacity of Mallard and Pekin ducks influences the response to estrogen treatment.

The reduction of the size of the testes of the Pekin drakes following estrogen administration in the first experimental series, but not in the second was probably a result of the fact that the testis weights were increasing during the period between the two autopsy dates, February 27 and April 10. They had probably reached their maximum breeding-season size at the beginning of the second experiment; the pituitary body at this season would probably be highly active (16) and more difficult to suppress by estrogen. Furthermore, the testis would probably be secreting its maxi-

mum amount of male hormone, and testosterone propionate has a protective action against suppression of testis size in pigeons receiving estrogens (17). At the earlier date, however, neither the hypophysis nor the gonads presumably had reached their maximum secretory activity and thus were more readily suppressed. The above explanation also holds for the Mallard drakes since in the first experiment the testes were almost minimal in size and thus could not be much further reduced, but by April 10 the testes were enlarging and at a stage somewhat similar to that reached by the Pekins in the first series and were, therefore, readily suppressed by estrogen.

Since the highest blood lipid values in the estrogen treated Mallard and Pekin ducks were obtained at the time the testes were enlarging rapidly, it may be possible to explain the difference in blood lipids which were observed in the controls, because the breeding cycles are not synchronized in the two breeds. It is interesting that, although there does not seem to be a very close correlation between the increase of serum calcium and that of serum fat, grossly apparent lipemia was never observed in a bird unless calcium values were extremely high.

The fact that within each of the two breeds of ducks increased serum calcium levels were roughly paralleled by increased formation of endosteal bone allows the conclusion that these two factors may be very closely correlated. It would be difficult, however, to explain the presence of extremely high levels of serum calcium at the same time that rapid proliferation of osteoblasts and deposition of calcium in the long bones occurs unless the retention of calcium is maximal since mobilization of calcium from other sources was not indicated. However, there may be another mechanism in operation since in mice (18) hyperossification of the long bones occurs quite readily under the influence of estrogens, and without appreciable increases in serum calcium levels. That the controlling mechanism in mice is different from that in birds is indicated by the effects of simultaneous administration of testosterone propionate with the estrogen. In mice the endosteal hyperossification is inhibited by this treatment (19) while in the Pekin drakes of the second experimental series, the failure of the testes to be reduced in size did not influence the resulting hyperossification. This lack of inhibition by the secretions of the testis in birds is further supported by the fact that in some unpublished preliminary experiments on pigeons and sparrows simultaneous administration of testosterone propionate with the estrogen did not alter the new endosteal bone formation in estrogen treated birds. In any case the rapid transfer of inorganic salts in the new endosteal bone indicates a high general level of calcium metabolism. It is difficult at this time to associate the response in the birds receiving estrogens with either the action of vitamin D sterols or parathormone.

While it is true that in the Pekin drake slight increases in serum calcium are produced by treatment with parathyroid extract, the fact that the extract did not alter the bone or the blood lipids indicates that the parathyroids do not play a decisive role in the changes which occur following estrogen treatment.

SUMMARY

Hyperossification of the long bones and an extensive rise in serum calcium followed the administration of estrogen to Mallard and Pekin drakes. The extent of new bone formation decreased proximo-distadly from the femur to the tarsometatarsus. The control values for calcium ranged from 8.64 mg.% to 10.40 mg.%, but with daily doses of 2 mg. estradiol benzoate for 21 days, these values rose to between 54.56 mg.% and 68.00 mg.%.

Blood lipids, inorganic phosphorus and phosphatase values also increased during the treatment with estrogen.

The Mallard and the Pekin drakes differed in their response to estrogen in that

the latter showed a much more extensive hyperossification than the former. The serum calcium values were only slighly lower for the treated Mallards than for the Pekins. The blood fat varied according to breed and probably to season as well as in response to estrogen injection.

The testes of both breeds of ducks were suppressed by the estrogen unless they were of maximal breeding season size or in the minimal nonbreeding condition. The adrenals and parathyroids were possibly somewhat enlarged, but none of the other endocrine glands were altered grossly by estrogen treatment. The liver enlarged and became yellowish in color, and acetone bodies were increased in the blood.

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THE EFFECT OF LONG TERM STIMULATION OF MALE AND FEMALE RATS WITH ESTRONE, ESTRADIOL BENZOATE, AND TESTOSTERONE PROPIONATE ADMINISTERED IN PELLET FORM[1]

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THERE HAVE BEEN numerous studies of the effects of fat-soluble sex hormones on the genital and endocrine organs of male and female rats (1, 2, 3). These results have been achieved by the use of daily or intermittent injections of the hormones in solution. Since Deanesly and Parkes (4, 5) described the use of large crystals or compressed tablets, a more suitable method has become available for this type of experimentation. This method has been applied to a study of the process of inactivation of these compounds in the animal body (6).

EXPERIMENTAL METHODS

We are especially interested in the efficacy of this mode of administration, and in the past two years have gathered data on the rate of daily absorption and the effect of estrone,[3] estradiol benzoate,[4] and testosterone propionate,[5] when administered as small compressed pellets. The physiological changes produced by these substances in male and female rats have been studied. Particular note of the long continued action on vaginal smears of female rats has been made. Change in body weights of the animals, and of the weight, gross and microscopic structure of the endocrine organs have been noted. Neoplastic changes were recorded when they occurred.

White rats of the Wistar strain and hooded rats of the McCollum strain were used. The animals were housed, 6 in a large cage, in a well lighted room, and fed on a standard ration of dog biscuits. The animals were killed by a blow on the head and then exsanguinated. Whenever possible the organs were weighed on a damped balance as soon as they were removed. The tissues were fixed in Bouin's fluid or Zenker-formol, embedded in paraffin, and stained with hematoxylin and eosin.

The pellets were made of estrone (ketohydroxy-estratriene), estradiol benzoate, and of testosterone propionate. The pure crystalline material is forced into a series of small holes conveniently arranged in a metal plate that is bolted to a larger plate. After the holes are well filled, the material is compressed by hammering a tightly fitting plunger into each hole. The pellets are removed by separating the plates and forcing each one out with the plunger. This method has been described in the preparation of insulin pellets (7). The pellets are inserted by placing them in a large bore needle which is then introduced into the desired site. The pellet is forced out of the needle by a tightly fitting stylet.

The pellets are readily located by palpation in the live as well as in the dead animal, and if necessary can be found by transilluminating suspected areas of skin. The thin encapsulating layer of connective tissue is easily stripped off. The pellets are then dried before weighing. The smaller the unabsorbed portion of the pellet, the greater is the possible error in calculating the amount absorbed, because of adherent bits of tissue and fragmentation of the pellet.

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[3] Supplied as theelin through the courtesy of Dr. Oliver Kamm of Parke, Davis & Co.

[4] Supplied as progynon-B through the courtesy of Dr. E. Schwenk of Schering Corporation.

[5] Supplied as perandren through the courtesy of Dr. Robert Mautner of the Ciba Pharmaceutical Products, Inc.

TABLE 1. ABSORPTION FROM ESTRONE AND ESTRADIOL BENZOATE PELLETS IMPLANTED SUBCUTANEOUSLY

	Rat No.	Original wt.	Wt. after removal	Amount absorbed	No. of days	Ave. amount absorbed per day
			Estrone Pellets			
		mg.	mg.	mg.		γ
In normal female rats	4	2.6	1.2	1.4	115	12.1
	4[1]	3.7	2.3	1.4	101	14.0
	5	2.0	1.4	0.6	115	5.2
	6	2.9	1.9	1.0	115	8.7
	6[1]	2.6	2.3	0.3	83	3.6
	62	1.1	0.4	0.7	216	3.2
	63	1.6	0.5	1.1	216	5.1
	64	1.3	0.0	1.3	216	6.0
	65	2.5	1.5	1.0	216	4.6
	46	1.2	0.7	0.5	233	2.1
	47	2.1	0.0	2.1	233	9.0
					Ave.	6.7
In castrate female rats	10	1.5	1.0	0.5	73	6.8
	11	1.8	1.5	0.3	32	9.4
	12	1.7	1.0	0.7	148	4.7
	13	1.4	1.1	0.3	55	5.6
	14	1.7	1.3	0.4	44	9.1
	15	1.8	1.1	0.7	115	6.1
	15[1]	1.5	0.8	0.7	120	5.8
	19	3.6	1.5	2.1	115	18.2
	20	2.9	1.8	1.1	115	9.5
	21	3.2	1.7	1.5	115	13.0
	22	2.5	1.4	1.1	115	9.5
	22[1]	0.9	0.4	0.5	80	6.2
	23	2.9	1.7	1.2	115	9.5
	23[1]	1.2	0.4	0.8	62	12.9
	49	1.4	0.0	1.4	230	6.1
	50	1.4	0.4	1.0	237	4.2
	51	1.9	0.8	1.1	220	5.0
					Ave.	8.3
					Total Ave.	7.7
			Estradiol Benzoate Pellets			
In normal female rats	44	2.7	1.5	1.2	196	6.1
	45	3.3	1.2	2.1	224	9.4
	48	3.0	2.5	0.5	69	7.2
	68	3.7	1.6	2.1	223	9.4
					Ave.	8.0
With testosterone propionate in normal female rats	59	2.7	1.1	1.6	203	7.8
	60	3.4	2.7	0.7	74	9.4
	61	3.3	2.0	1.3	206	6.3
					Ave.	7.8
With testosterone propionate in normal male rats	436	3.0	0.8	2.2	223	9.9
	437	3.3	1.7	1.6	223	7.2
	438	2.8	1.2	1.6	223	7.2
	439	3.5	0.2	3.3	223	13.3
	440	3.3	1.3	2.0	223	8.9
					Ave.	9.3
					Total Ave.	8.5

[1] Second pellet.

RESULTS

A. *Estrone Pellets in Normal Female Rats*

Ten intact female rats (No. 4, 5, 6, 46, 47, 62–66) received pellets of estrone and except for one which died after 27 days with pyometria, the other 9 died or were killed after 115 to 233 days. The pellets of rats 4, 5, and 6 were removed after 115 days, cleaned, dried and weighed, and the absorption of hormone per day was calculated. The others had their pellets removed at autopsy. In all cases the pellets were fairly hard and retained their original shape. Absorption appeared to be uniform on all aspects. The duration of pellet action, original weight of pellet, final weight of pellet, and average absorption per day are recorded in table 1. From 2.1γ to 14γ are absorbed per day, an average of 6.7γ per day for 11 observations.

TABLE 2. BODY AND ORGAN WEIGHTS OF NORMAL FEMALE RATS WITH ESTRONE PELLETS

Animal No.	4	6	62	63	64	65	66	46	47
Body wt. at beginning, gm.	234	270	47	42	48	44	150	166	128
Body wt. at end, gm.	300	210	228	194	200	166	134	174	174
R. ovary, mg.			34.9	19.5	21.5	16.7		30.7	54.0
L. ovary, mg.			41.3	33.5	19.6	12.2		31.0	44.0
Uterus, mg.			472	353	376	444		875	598
R. adrenal, mg.			37.7	36.3	30.3	27.1		31.5	37.0
L. adrenal, mg.			33.8	39.4	35.6	23.2		30.2	37.0
Thymus, mg.			194	153	252	88		92	93
Hypophysis, mg.	36.2	31			7.5	5.4	9.0	10.2	14.7
Thyroid, mg.			14.3	15.3	17.5	10.8		14.7	15.0

Vaginal smears were observed daily over an average period of 160 days. They showed profound alteration of the estrous cycle with massive clumps of cornified cells appearing for long periods. Irregular intervals in which the smear showed combinations of leucocytes, epithelial cells and cornified cells alternated with a purely cornified smear. In two animals, 4 and 6, removal of the pellet allowed a normal cycle to appear in 4 days, followed by a prolonged period of estrus. Button (8) has described similar findings. The third animal, 5, died of marked pyometria shortly after removal of the pellet.

All the pellets removed from these animals were reimplanted in test spayed females; within 2 days cornified epithelium replaced the leucocytes. This showed that the pellets still retained their activity.

At autopsy only rats 4 and 6 had large hypophyses; all had large adrenals. No apparent change occurred in thyroid weights. Compare weights in table 2 with those of normal control series in table 3.

Microscopically there was marked chromophobe hyperplasia in the hypophysis. Animals 4, 5, 6 and 66 died with pyometria. The uteri showed an intense leucocytic infiltration. In many instances there was early organization of the inflammatory reaction. The adrenals were large with narrowing of the cortical layers and prominent medulla but no necroses were found. Liver, spleen and pancreas appeared normal. The ovaries showed follicles but no evident corpora lutea. There was focal and diffuse squamification of uterine epithelium with areas of cystic dilatation of the glands. Glands were found deep in the myometrium which was greatly thickened with marked hyalinization and fibrosis, and infiltration of numerous leucocytes and eosinophils. Marked keratinization of the vagina and cervix was also present with sheets of cells continuous with the cervix. *Rat 5* showed hyperplastic changes in the breast with numerous small acini lined by columnar epithelium growing everywhere; but

the cells did not themselves appear abnormal. Breast sections were not taken from other animals because no gross changes were apparent.

Such changes have been described previously (3), but in all previous reports the prolonged stimulation was obtained by daily or intermittent injections of hormone in oil. These results were obtained with one pellet in each of 7 animals, and with 2 pellets in each of 2 animals. The feasibility of this method for prolonged stimulation is apparent if one compares the changes that have been described in the uterus, breast and cervix with those obtained by daily injections of the solution. This work has been admirably reviewed by Gardner (12).

B. Estradiol Benzoate Pellets in Normal Female Rats

Four intact female rats (*44, 45, 48, 68*) received pellets of progynon-B. They died after 69 to 244 days. The original and final weights of the pellets and absorption per

TABLE 3. BODY AND ORGAN WEIGHTS OF ADULT FEMALE CONTROL RATS

Animal No.	C 101	C 102	C 103	C 104	C 105	Ave. weight
Body wt., gm.	200	190	180	170	171	182
R. ovary, mg.	53.5	64.0				
L. ovary, mg.	26.4	29.2	41.7[1]	40.0[1]	62.3[1]	31.7[1]
Uterus, mg.	414	421	493	331	351	402
R. adrenal, mg.	17.0	21.2	25.1			
L. adrenal, mg.	25.2	28.1	28.6	60.0[1]	63.0[1]	26.8[1]
Thymus, mg.	92	231	280	154	162	184
Hypophysis, mg.	4.8	8.9	8.8	7.0	8.2	7.5
Thyroid, mg.	13.5	18.7	16.2	13.4	14.8	15.3

[1] For each ovary and adrenal.

day are recorded in table 1. The average absorption from the pellets in this group was 8.0γ per day.

Vaginal smears were observed daily over an average period of 168 days. Here, as with estrone, cornification appeared for long periods, but there were more prolonged anestrous phases with leucocytes and absence of squamous cells. The pellets were active in castrate females after removal. The ovaries were about normal in weight. The uterus of *45*, the only one weighed, was twice normal. The average adrenal and thyroid weights were normal. The hypophyses were enlarged; that of *rat 48* weighed 20 mg.

Animals *44, 48* and *68* died with uterine infection. *Rat 45* had hyperplastic small acini growing everywhere in the breast. *Rat 68* had a localized adenocarcinoma in all breasts with marked squamification of the ducts. The uteri showed cystic dilatation of the glands with mucification and infection, but no obvious squamification. Ulcerated vagina and cervix with an atrophic scarred uterus was found in *rat 68*.

These changes are similar to those produced in the previous group, except for absence of squamification of the endometrium.

C. Estrone Pellets in Castrate Female Rats

Fourteen castrate female rats (*10–15, 19–23, 49, 50, 51*) had pellets of estrone implanted for 32 to 237 days. The pellets of *15, 19, 20, 21, 22* and *23* were removed after 115 days and new pellets were inserted.

The data on duration of pellet action, original and final weight of pellets and absorption per day are recorded in table 1. The average absorption for 17 observations was 8.3γ per day.

The vaginal smears were observed daily over an average period of 154 days. Cornified smears persisted throughout except for occasional leucocytes and epithelial cells. Two days after removal of the pellets, vaginal smears reverted to the pure leucocytic type, typical of the castrate; two days after insertion of the new pellets the smears again showed cornified cells.

The adrenals and hypophyses of this group were definitely enlarged. Hypophysis of *rat* 22 weighed 40 mg.; that of *rat* 20 weighed 35.8 mg. (table 4).

TABLE 4. ADRENAL AND HYPOPHYSIS WEIGHTS IN CASTRATE FEMALE RATS WITH ESTRONE PELLETS

Animal No.	10	11	12	13	14	15	20	21	22	23
Both adrenals, mg.	157		76	75			140	115	103	94
Hypophysis, mg.	30.1	20.7	29.6	16.5	17.1	23.9	35.8	18.5	40.0	15

Microscopically almost all the uteri showed evidence of infection with organisms, and eosinophils infiltrating the uterine musculature. There was cystic dilatation of the uterine glands with focal areas of metaplastic change and growth of the glands through the myometrium. The changes in the castrates were more marked than those occurring in the intact group, and intense scarring of the myometrium was also present with marked squamification in the vagina and cervix. The adrenals were all large with narrowed cortex but no reticular necroses.

The parathyroids appeared prominent. The thyroid, although not grossly enlarged, showed enlarged acini, filled with colloid. The lining cuboidal cells were plump and regular, lining the basement membranes. There was no scarring or lymphoid infiltration, and no evidence of neoplasia.

D. Testosterone Propionate Pellets in Normal Female Rats

For 36 days. A group of 5 normal females (534–538) received pellets of testosterone propionate and were killed 36 days later to see the effects of continuous stimulation without permitting restitution to take place in the genital apparatus. The average absorption of testosterone propionate from 5 pellets was 54γ per day over a 36-day period. The pellet data are recorded in table 5. The vaginal smears were predominantly leucocytic throughout the duration of the experiment; no distinct estrous smears appeared.

All animals gained weight except 538, but the ovaries, uteri, adrenals and hypophyses were obviously small. Thyroid weights were normal (table 6). The microscopic changes were similar in all the animals of this group. The adrenals were small. The ovaries were small and showed no corpora lutea (fig. 1). The uteri showed no squamification or mucification except for 536 which showed some mucification of the uterine glands. The thyroid acini of *rats* 534 and 535 were enlarged and filled with colloid. No sections were taken of the other thyroid glands in this group.

For 231 days. This group of 5 normal females (52–56) had pellets of testosterone propionate inserted 231 days before death (table 7). These animals showed prolonged diestrous smears interspersed with short periods of 4 to 5 days when cornified cells with some leucocytes predominated. Smears were examined daily over this period. The average absorption from testosterone propionate pellets is 59γ per day, so that the pellets were probably effective for less than 50 days (table 5). This allowed a period of about 180 days for restitution to take place after this substance was gone. It is interesting that squamous cells did not reappear in the smears until more than 60 days after it was calculated that the testosterone propionate was completely absorbed.

TABLE 5. TESTOSTERONE PROPIONATE PELLETS

Rat No.	Sex	Castrate or intact	Original wt.	Wt. after removal	Amount absorbed	No. of days	Pellet site	Amount absorbed per day
			mg.	mg.	mg.			γ
534	F	I	1.9	0.1	1.8	36	Subcut.	50
535	F	I	4.0	1.5	2.5	36	Subcut.	69
536	F	I	3.8	1.8	2.0	36	Subcut.	55
537	F	I	3.0	1.3	1.7	36	Subcut.	47
538	F	I	3.7	2.0	1.7	36	Subcut.	47
7	F	I	5.9	0.5	5.4	115	Subcut.	49
8	F	I	5.1	1.5	3.6	53	Subcut.	68
9	F	I	3.6	1.2	2.4	53	Subcut.	45
17	F	C	6.6	1.2	5.4	115	Subcut.	45
18	F	C	5.8	0.5	5.3	115	Subcut.	46
439	M	I	2.2	1.3	0.9	15	Subcut.	60
444	M	I	3.7	1.0	2.7	40	Subcut.	68
467	M	C	2.5	1.2	1.3	21	Subcut.	61
469	M	C	3.0	1.7	1.3	21	Subcut.	62
472	M	C	2.5	1.0	1.5	21	Subcut.	71
441	M	C	3.6	1.8	1.8	21	Spleen	85
450	M	C	2.8	1.6	1.2	21	Spleen	50
451	M	C	1.9	0	1.9	21	Spleen	57
452	M	C	3.6	2.1	1.5	21	Spleen	75
453	M	C	3.2	1.2	2.0	21	Spleen	60
454	M	C	2.3	1.6	0.7	21	Spleen	35
462	M	C	3.8	2.3	1.5	21	Spleen	71
463	M	C	3.4	1.1	2.3	33	Spleen	70
464A	M	C	3.1	1.7	1.4	21	Spleen	67
465	M	C	3.2	2.1	1.1	21	Spleen	52
474	M	C	4.1	2.7	1.4	21	Spleen	67
475	M	C	4.5	2.8	1.7	21	Spleen	73
452	M	C	3.7	0.7	3.0	40	Liver	75
468	M	C	1.7	0.9	0.8	21	Liver	40
470	M	C	1.8	0.9	0.9	21	Liver	43
461	M	C	3.7	0.7	3.0	40	Kidney	75
466	M	C	1.5	0.2	1.3	21	Kidney	62
471	M	C	1.4	0.3	1.1	21	Kidney	52

Experiments with pellets in spleen, liver and kidney reported in (6)
Ave. pellet absorption for intact rats 55.8γ
Ave. pellet absorption for castrate rats 60.5γ
Total Ave. 59.0γ

The organ weights were approximately normal (table 7). Microscopically no significant changes were found. The ovaries contained corpora lutea (fig. 2, cf. fig. 1).

Mazer and Mazer (9) have found decreases in pituitary, adrenal, ovarian and uterine weights of the immature rat, with inhibition of estrus and mucification of the vaginal epithelium following testosterone propionate. The adult rat reacted similarly

TABLE 6. BODY AND ORGAN WEIGHTS OF NORMAL FEMALE RATS WITH TESTOSTERONE PROPIONATE PELLETS FOR 36 DAYS

Animal No.	534	535	536	537	538
Body wt. at beginning, gm.	126	90	120	120	120
Body wt. at end, gm.	180	178	170	180	120
R. ovary, mg.	11.9	7.9	10.0	9.3	8.1
L. ovary, mg.	10.9	11.1	10.7	6.6	6.6
Uterus, mg.	148	156	232	245	115
R. adrenal, mg.	18.5	17.6	14.3		15.5
L. adrenal, mg.	18.0	13.0	13.8	13.3	15.3
Thymus, mg.	205		191	115	114
Hypophysis, mg.	6.4		7.1		6.0
Thyroid, mg.	14	13.5	16.0	12.0	

TABLE 7. TESTOSTERONE PROPIONATE IN NORMAL FEMALE RATS FOR 231 DAYS

Animal No.	52	53	54	55	56
Duration of implantation, days	231	231	231	231	231
Pellet wt. on implantation, mg.	2.4	2.3	2.8	2.8	2.4
Body wt. at beginning, gm.	50	46	48	45	42
Body wt. at end, gm.	222	242	248	190	176
Both ovaries, mg.	57.5	77.0	49.4	65.4	34.3
Uterus, mg.	372	656	340	365	224
Both adrenals, mg.	59.5	54.4	49.6	36.5	43.1
Thymus, mg.	232	190	154	285	117
Hypophysis, mg.	9.3	12.0	10.3	7.9	7.5
Thyroid, mg.	13.4	13.4	16.3	10.0	13.4

to the androgen, except that there was no significant decrease in pituitary weight. They found that these effects were temporary and restitution of structure and function occurred within 29 days. Their criteria of return of function was follicular growth, ovulation and luteinization.

Function as judged by return to normal estrous cycles did not occur in our animals for 60 days after the hormone was absorbed.

E. Estradiol Benzoate and Testosterone Propionate in Normal 30-day-old Female Rats

Testosterone propionate pellets weighing from 2.6 to 3.6 mg. and estrone pellets weighing from 2.9 to 3.4 mg. were simultaneously placed in five normal 30-day-old females (57–61). Within 2 days the smears became squamous in type and remained so persistently, except for rare leucocytic smears, throughout the duration of the experiment. Estradiol benzoate pellets remained. The data for pellets of *rats* 59, 60 and 61 are recorded in table 1. The absorption was the same as when progynon was given alone. The testosterone propionate pellets were completely absorbed except in *rat* 58. The hypophysis of *rat* 60 weighed 21 mg. The adrenals of *rat* 58 together weighed 54.8 mg. *Rats* 57, 58 and 60 had uterine infection with peritonitis and ovarian abscesses.

When both testosterone propionate and estrone were given together, the estrone effect was predominant and the vaginal smears showed this effect by squamification. Enlargement of the hypophysis occurred in *rat* 60.

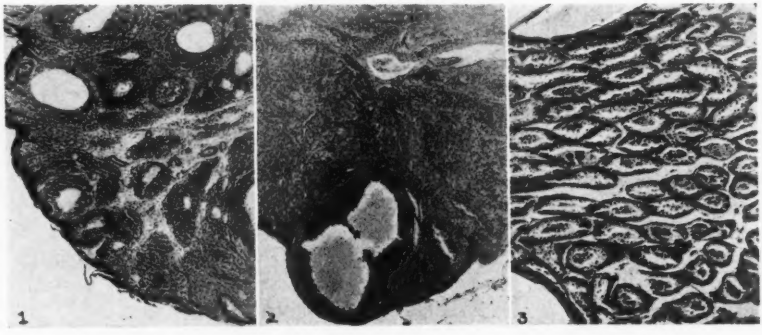

FIG. 1. TESTOSTERONE PROPIONATE PELLETS IN NORMAL FEMALE RATS FOR 36 DAYS (535). Ovary small with atretic follicles and no corpora lutea. Fig. 2. TESTOSTERONE PROPIONATE PELLETS IN NORMAL FEMALE RATS FOR 231 DAYS (52). Ovary is normal in size and contains well developed corpora lutea. Fig. 3. TESTOSTERONE PROPIONATE AND PROGYNON PELLETS IN 30-DAY-OLD MALE RATS (438). Testis is atrophic and shows degenerated seminiferous tubules and absence of spermatozoa.

F. Estrone and Testosterone Propionate in Previously Pregnant Female Rats

Similarly, in this group of 4 previously pregnant rats (40–43), theelin pellets from 2.2 to 3.7 mg. were inserted simultaneously with testosterone propionate pellets. The animals were observed over an average period of 238 days. The smears always showed squamous or epithelial cells but no leucocytes. Microscopically *rats* 40 and 41 showed only a shell of adrenal cortex, and mucified and cystic uterine glands. Ovaries were tiny and showed no corpora lutea but only infantile follicles. No squamification was found in the uterus.

The effect of testosterone propionate was completely masked by the persistent action of the estrogen. In the dosages used, however, the estrogen persisted long after testosterone propionate was completely absorbed. Whether testosterone propionate augmented the estrogen effect while it was present is impossible to say from these studies. Zuckerman (13) however, has shown that 50 mg. doses of testosterone propionate given 8 days apart counteracted the effect from 0.1 mg. estrone given daily for 10 to 21 days in immature male rhesus monkeys. In these rats, absorption of 60γ of testosterone propionate did not counteract 8γ of estrone per day.

G. Testosterone Propionate in Adult Castrate Female Rats

The typical castrate smear was unchanged by testosterone propionate in 4 rats (16–18, 29). After 115 days the pellets of *rats*, 16, 17 and 18 were removed. The leucocytic smear persisted and new pellets were inserted. The average absorption of testosterone in this group was 45γ per day. The pellet data for *rats* 17 and 18 are recorded in table 5. This group was followed over an average period of 175 days. The hypophyses of *rats* 16 and 17 weighed 10.4 and 11.1 mg. respectively. The adrenals of *rat* 17 weighed 55.5 mg. together. Microscopically *rat* 16 showed colloid filled thyroid acini with plump cuboidal cells but there were no other changes in the gland. The uterine glands were tortuous and showed mucification. The adrenals were small.

The lack of any cornification of the vagina with testosterone propionate would be expected.

H. Testosterone Propionate and Estradiol Benzoate in 30-day-old Male Rats

Five 30-day-old males (436–440) received pellets of progynon and testosterone propionate simultaneously. The testosterone propionate pellets weighed 2.2 to 3.6 mg. and the progynon pellets weighed 2.8 to 3.5 mg. The progynon data are recorded in table 1. Testosterone propionate remained only in *rat* 439 which died after 15 days. The other rats were killed after 223 days. The atrophy of the testes was striking, each weighing only 97 mg. or about one-tenth the amount noted in adult males with testosterone propionate alone (see next section). The average weight of the prostates was 110 mg. The seminal vesicles too, were atrophic. The hypophysis of *rat* 437 is enlarged, weighing 29.9 mg. The adrenals were definitely enlarged when compared to normal and castrate males with and without testosterone propionate; the average weight of each being 32 mg. All the data for comparison are recorded in table 8. Microscopically the testes showed degenerated seminiferous tubules with cast off spermatogenic elements in the lumina and absent spermatozoa. The thyroid weights were normal. *Rat* 438 showed a small colloid nodule in the thyroid; *rat* 439 showed small acini filled with colloid. No other thyroid sections were taken. The urethra was squamified and the prostate showed normal epithelium.

Pfeiffer and Gardner (10) reported testicular degeneration with estrogens in pigeons. This was partially or completely inhibited by simultaneous use of testoster-

TABLE 8. TESTOSTERONE PROPIONATE IN MALE RATS

		Pellets implanted for days								
		60	70	80	90	100	110	120	130	140
		Body wt., gm.								
Castrate	B	102[1]	105	112	112	96	90	192	162	180
	E	206[1]	206	298	298	270	216	264	266	252
Castrate with testosterone	I[2] B	110	115	110	100	95	93	154	184	175
	I E	186	240	204	360	250	246	300	310	216
	II[2] B	114	112	132	111	96	95	200	184	170
	II E	220	208	202	300	330	236	310	310	316
Normal with testosterone	B	132	105	132	102	95	95	208	206	200
	E	196	228	254	304	260	250	262	350	365
Normal	B	214	196	204	218	188	196	234	234	250
	E	222	196	218	246	270	262	300	304	370
		Pellet wt., mg.								
Castrate with testosterone	I B	2.6	3.1	2.3	2.9	3.3	3.8	4.5	3.8	4.4
	I E	0.8	0.5	0	0	0.4	0	0	0	0
	II B	2.4	3.1	2.1	2.6	3.3	3.7	4.4	4.1	4.5
	II E	1.0	0.6	0	0	0	0	0	0	0
Normal with testosterone	B	2.2	3.1	4.1	2.6	3.2	3.4	4.1	4.6	4.5
	E	0	0.7	0.9	0	0.3	0	0	0	0
		Av. absorp. in mg.								
		1.5	2.5	3.2	2.7	2.9	3.6	4.3	4.2	4.5
		Absorp. per day in γ								
		27	36	40	30	29	33	36	32	32
SEMINAL VESICLE		*Organ weights in mg. per 100 gm. of body weight*								
Castrate		3	5	4	4	4	2	18	18	11
Castrate with testosterone	I	156	86	51	34	67	47	41	32	11
	II	81	85	43	50	32	53	47	45	62
Normal with testosterone		124	147	155	100	147	151	120	152	132
Normal		98	85	151	94	58	130	125	137	119
TESTIS										
Normal with testosterone		1118	724	683	530	582	800	716	793	685
Normal		950	1291	1113	1190	952	942	900	954	873
HYPOPHYSIS										
Castrate		4.4	2.8	3.5	5.2	4.4	5.4	5.8	5.3	5.8
Castrate with testosterone	I	3.5	4.5	4.0	4.1	3.7	4.9	3.6	4.4	2.4
	II	4.6	3.1	6.1	4.1	4.0	4.2	4.0	4.4	4.4
Normal with testosterone		2.9	2.7	3.1	2.7	2.2	2.8	3.1	3.1	3.1
Normal		3.0	3.3	3.3	3.1	2.9	2.0	3.0	3.2	3.4
PROSTATE										
Castrate	D-V[3]	26	33	18	21	28	30	36	55	39
Castrate with testosterone	I D	123	58	62	36	71	57	52	33	63
	I V	168	100	88	63	105	83	103	49	74
	II D	115	84	48	52	37	45	41	61	51
	II V	148	175	67	59	45	39	116	52	110

[1] B—beginning of experiment; E—end of experiment
[2] I, II—indicate the two rats of this group.
[3] D—dorsal lobes; V—ventral lobes

TABLE 8—*Continued*

		Pellets implanted for days								
		60	70	80	90	100	110	120	130	140
Normal with testosterone	D	112	104	136	66	121	155	121	155	97
	V	104	171	162	96	102	163	176	236	148
Normal	D	94	100	119	120	89	79	78	119	95
	V	59	78	146	133	104	59	98	133	161
ADRENAL										
Castrate		20.2	14.0	8.4	9.1	10.3	18.3	11.7	7.9	10.6
Castrate with testosterone	I	12.9	12.8	14.2	11.2	8.9	10.1	7.6	10.8	10.2
	II	11.6	12.3	18.0	13.7	8.8	12.0	9.4	9.7	8.7
Normal with testosterone		17.2	14.2	10.2	10.0	12.3	10.8	11.5	10.4	7.2
Normal		15.9	15.7	11.6	9.1	11.2	7.3	10.0	7.6	7.1
PENIS										
Castrate					22	19	26	60	54	45
Castrate with testosterone	I				58	77	86	66	62	95
	II				67	60	68	83	77	79
Normal with testosterone					73	81	88	88	88	75
Normal					91	75	139	75	74	56
PREPUTIAL GLAND										
Castrate		20	18	10	7	15	17	13	8	16
Castrate with testosterone	I	27	28	14	10	20	15	24	28	31
	II	25	33	24	19	19	16	26	29	39
Normal with testosterone		44	45	31	31	32	45	26	16	32
Normal		23	25	25	19	29	22	23	23	19
THYMUS										
Castrate			189	91	98	142	170	76	95	76
Castrate with testosterone	I		114	136	155	161	114	70	80	41
	II		139	170	95	110	102	51	58	89
Normal with testosterone			100	78	136	58	70	66	78	41
Normal			67	177	83	94	41	85	90	98
THYROID										
Castrate		6.8	8.6	5.6	7.0	8.0	9.6	7.2	7.5	4.8
Castrate with testosterone	I	7.8	7.1	9.0	6.5	5.9	8.0	7.1	6.4	12.8
	II	6.5	8.0	9.1	8.3	7.9	5.9	7.5	6.6	7.1
Normal with testosterone		6.9	9.1	7.4	8.7	8.6	5.7	7.9	4.4	4.8
Normal		7.0	6.8	7.8	7.1	7.6	6.5	7.2	8.8	6.5

one propionate. Kun and Peczenik (11) have reported a great augmentation of the response to the ejaculation test, when estrogen was given with the male hormone. These experiments, however, are not analogous, since, except for *rat* 439 which died after 15 days with testicular degeneration despite testosterone propionate, the others could not have had testosterone propionate remaining in any case for more than 60 days. The largest testosterone pellet weighed 3.6 mg. and the average absorption is 59γ per day. The estrone effect predominated in the genital organs at autopsy, with enlarged adrenals and hypophyses and marked degeneration of the testes, and absence of spermatozoa (fig. 3).

I. Testosterone Propionate Pellets in Male Rats

A total of 45 male rats were used in this experiment. There were 9 groups of animals, each group containing 5 rats, namely: one castrate control, two castrates, each

with a testosterone propionate pellet, one normal with a testosterone propionate pellet, and one normal control. The rats were weighed at the onset of the experiment. One group of 5 animals was killed after 60 days, and an additional group was killed after each succeeding 10-day period. This gave for comparison 9 groups, which were followed for 60, 70, 80, 90, 100, 110, 120, 130 and 140 days. In table 8 the organ weights of these animals are recorded. There are too few animals to draw any widespread conclusions. However, from a consideration of these weights and also from an analysis of the weights of some of these organs per 100 gm. of body weight (table 8), certain suggestive data are obtained.

The kidney weights of the castrates seem less than those of the other 3 groups. The penis weights of the castrates were definitely smaller, but in the groups that went 120 to 140 days this atrophy was not so marked. These rats were quite heavy, however, at the time of castration. The androgen maintained the penis weight satisfactorily in the castrates, but caused no marked increase in the weight of the normal rat's penis.

Atrophy of the bulbourethral muscles occurred in the castrate, but the weights were maintained with testosterone propionate. The normal too increased in weight with the hormone. The preputial glands did not show as striking atrophy after castration as the other genital organs and the androgen did not restore these weights to normal as readily as in the case of the other genital organs. The normal preputial glands were stimulated beyond the controls with testosterone propionate.

The weight of the heart was not altered by castration or the addition of androgen.

No significant variations could be found in the adrenal weights of this small series after 60 to 140 days. These adrenals, however, all weighed less than those of the female rats.

The hypophyses of the normal rats receiving androgen seemed smaller for the most part than the normal controls. Definite enlargement was still apparent in castrates receiving testosterone propionate pellets, but the castrates had the largest hypophyses (table 8).

No appreciable variation in thyroid weights was found.

A great variation in splenic weights was recorded, but these showed no definite correlation to castration or hormone administration.

The testes of the normal rats receiving androgen were all significantly smaller than those of the normal controls, except those that went 130 and 140 days. Here absorption of all the hormone before termination of the experiment might explain the difference.

The rats all gained weight; some doubled or tripled their original body weights during the course of the experiment. No significant increase in weight over the controls occurred in the testosterone propionate treated animals. The differences between normal with the androgen, and normal controls is due to the fact that the controls were first weighed only a short time before the termination of the experiment.

The seminal vesicles of the normal animals with testosterone propionate weighed more than those of the normal controls. The castrate seminal vesicles showed marked atrophy, and those of the castrates with androgen showed significant stimulation, but the weights were not as great as those of the normal controls.

The prostates of the castrates showed marked atrophy; in these, the dorsal and ventral lobes were weighed together. The castrates receiving androgen showed marked stimulation of dorsal and ventral lobes, but the ventral lobes in practically all cases weighed more than the dorsal lobes. The normal rats receiving testosterone propionate, on the whole, had larger prostates with heavier ventral lobes (table 8).

Sections were made from the organs and these were studied microscopically. The

organs of the castrate showed typical castrate changes with atrophy of the genital organs. The literature on these changes is completely summarized by Moore in his section on the biology of the testes (1). Castration cells were found in the hypophyses. These changes were not found in the genital organs of the castrates receiving androgen. No significant changes were noted in the spleen, liver, pancreas, kidneys, thymus or heart. The testes of the normal animals receiving testosterone propionate all showed the presence of spermatozoa. The adrenal changes were inconstant, but narrowed cortical zones were seen in some of the adrenals of the castrates and normals receiving androgen. Eosinophilic leukocytes were noted in many of the preputial glands.

The normal and castrate thyroids were composed of small acini lined with cuboidal epithelium and containing colloid. The thyroids of all rats that received testosterone propionate contained acini of varying size lined with cuboidal epithelial cells and filled with colloid. There were small colloid nodules in several of these glands. Stephens (14) has reported large colloid-filled acini in undernourished guinea pigs and explains the changes as due to decreased secretion of the pituitary thyrotropic principle. The thyroid changes in these experiments may also be due to hypophyseal inhibition caused by the estrogens and androgens used.

The weights of the pellets inserted are also recorded and also the weights of fragments recovered. Unfortunately, the pellets were small, so that either no hormone remained or the amount was too little to weigh accurately. The weights, however, are included in table 8. The average absorption from these pellets was 33γ per day. This is much less than the absorption of testosterone propionate recorded in table 5, but the latter data are undoubtedly more accurate.

J. Absorption of Estrone, Estradiol Benzoate and Testosterone Propionate from Pellets

A summary of the pellet data is recorded in tables 1 and 5. The average daily absorption from 28 theelin pellets was 7.7γ and the average absorption from 12 progynon pellets was 8.5γ. The average daily absorption from 33 testosterone propionate pellets was 59γ, so that the rate of absorption by this method is about 7½ times greater for testosterone propionate than for theelin or progynon. Several other points are also of interest. The absorption rate was not altered by the site, whether it be the subcutaneous tissue, spleen, liver or kidney. Absorption also takes place with the same rapidity from castrate and intact animal alike so that physiological need plays no role in the absorption. Variations in absorption are thought to be due to differences in the degree of compression of the substance composing the individual pellets and the steadily decreasing surface area exposed to the tissue fluids. For the continuous and uniform administration of these and related substances this method is convenient, accurate and economical. It also obviates the necessity for frequent handling of the animals for daily subcutaneous injections. These factors alone can influence body weight, and the repeated local tissue damage may be undesirable.

SUMMARY

Changes that occur in the endocrine and genital organs of male and female rats under the influence of pellets of estrone, estradiol and testosterone propionate have been described. Cornified smears persisted for long periods in intact females with estrone pellets interspersed with short periods of anestrus. Estradiol pellets produced similar alterations. Precancerous breast changes occurred in one rat after 115 days; definite adenocarcinoma was found in the breasts of another after 223 days.

Castrate female rats showed continuous vaginal cornification from an average of 154 days to as long as 230 days following the insertion of one estrone pellet. This group showed large colloid filled thyroid acini with cuboidal lining cells.

Adult female rats receiving testosterone propionate pellets for 36 days showed diminution of ovarian, uterine, adrenal and hypophyseal weights with inhibition of ovarian cycles and absence of corpora lutea.

Another group that received testosterone propionate pellets for 50 days followed by a period of 180 days to allow restitution to normal, showed anestrous smears for more than 60 days after the calculated disappearance of the hormone. At the termination of the experiment, corpora lutea were present in the ovaries and the organs were normal in weight and microscopic structure.

Estradiol benzoate and testosterone propionate given simultaneously to 30-day-old females produced squamification of the vaginal smears for an average period of 127 days. The combination did not affect the rapidity of estradiol absorption. The hypophysis proceeded to enlarge. The same effect occurred in previously pregnant females with estrone and testosterone propionate. The ovaries showed infantile follicles and no corpora lutea. Testosterone propionate did not alter the anestrous state of the castrate female rat.

Testosterone propionate and estradiol in 30-day-old males resulted in marked testicular atrophy. The testicles weighed only one-tenth the amount noted with testosterone propionate in adult males, and there was marked atrophy of the spermatogenic elements. The adrenals enlarged, as did the hypophysis of one. One showed a colloid nodule of the thyroid gland.

A series of 45 male rats that received testosterone propionate pellets were killed at intervals from 60 to 140 days and the changes that occurred in the organ weights and microscopic structure were analyzed. Each group included castrates and normals with and without testosterone propionate.

Finally the data on pellet absorption are summarized. About 59γ of testosterone propionate was absorbed per day and only 7.9γ of estrone. The rate was not affected by the site of introduction, the physiological need of the animal or the simultaneous introduction of both pellets.

Since completing these experiments, we have, in conjunction with Dr. H. G. Bennett treated a series of women with menopausal symptoms, using estrone pellets with very encouraging results. This report appeared recently (15).

We are indebted to Dr. W. G. MacCallum for his kind interest in this problem.

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THE RATE OF ABSORPTION AND EFFECTS OF TESTOSTERONE PROPIONATE PELLETS ON MICE

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THE EXPERIMENTAL ADMINISTRATION of androgens in oil by injection presents two major difficulties: *a*) the repeated injections are time consuming; *b*) the usual vehicle, sesame oil, possesses objectionable properties. Concerning the latter, it has been noted (unpublished data) that when androgen in this oil is injected into mice, the rate and amount of oil absorption is small and a sub-dermal accumulation of oil results. This condition is most undesirable since recent findings (1) indicate that toxic substance(s) may be present in the oil. A possible means of overcoming these undesirable properties was provided by the experiments of Deanesly and Parkes (2), who found that pure crystalline androgens were very effective when implanted subcutaneously as tablets.

METHODS

Assay. Many workers (cf. 3) have used the response of the seminal vesicles and prostate of the castrate rat to androgen therapy as a means of assay. On the other hand, investigators have given little attention to the response of these organs in the mouse. Deanesly and Parkes (4) found that similarly to those of the rat, the accessory sex organs of the mouse regressed to a basal state within one month following castration. They also found that the final weights of the organs are dependent on the age of the animals at time of castration: the older the mouse, the greater the size of the prostate and seminal vesicles. In a later study (5) Deanesly concludes that the mouse is not as satisfactory as the rat for quantitative purposes, since the larger animal gives a much greater response per milligram of androgen. In a similar comparison we have found that the rat gives an approximately 500% greater accessory sex organ weight per milligram of testosterone propionate absorbed.

Mice. In preliminary experiments Rockland mice from a stock colony were used. In the series of experiments presented here, greater uniformity of response was obtained by using an in-bred strain of mice, the Buffalo-Marsh.[1] These animals proved to be very satisfactory for assay purposes, the mice of each group demonstrating good agreement among their respective organ weights.

Pellets.[2] Deanesly (5) has reported two preliminary experiments with mice, using testosterone propionate pellets of 0.9 and 2.1 mg. from which 0.7 and 0.6 mg. respectively were absorbed after 10 days of implantation. Since we desired to extend the therapy over a period of months, we were interested in the feasibility of larger pellets. In the preliminary experiments, 10.0 mg. cylindrical pellets, 1.4 mm. diameter and 6.0 mm. length, were used. These were implanted subcutaneously in the right side near the shoulder by means of a No. 13 trocar. Although these pellets were implanted with a minimum of trouble, the mechanical difficulties involved in their preparation made them impractical. Therefore, in the next series of experiments more

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[1] These animals were made available by the courtesy of Dr. W. S. Murray of the Biological Station, Springville, New York.

[2] These pellets were presented under the trade name, perendren, by the Ciba Pharmaceutical Products, Inc. through the kind cooperation of Dr. E. Oppenheimer.

easily prepared, disc-shaped pellets were implanted. These were provided in two weights: *a*) 21.8 to 24.1 mg., average surface area 44.8 sq. mm. and *b*) 9.8 to 12.6 mg., average surface area 27.3 sq. mm. The diameter of both groups was the same, 3.23 mm., and the average thickness 2.89 mm. and 1.72 mm. respectively. These pellets were implanted by etherizing the mice, shaving the right side, making a small incision and inserting the pellet away from the opening into the shoulder region. The incision was closed with a single suture. The pellets were palpable during the course of the experiment.

All the pellets were weighed to 0.1 mg. on a torsion balance before and after the experiment. At autopsy the encapsulated pellet was exposed by cutting the skin at the right flank and peeling forward. After nicking the capsule, the pellet was extruded, washed in distilled water and dried on filter paper in a vacuum dessicator.

Potassium determinations.[3] Since testosterone propionate produces a positive urinary potassium balance in human hypogonads (6), it seemed worthwhile to determine whether this androgen also increased plasma potassium. If so, this effect might be interpreted as the cause of toxicity of androgens to adrenalectomized rats (7) because it is known (8) that alterations in the plasma potassium upset the animal economy. In the first few experiments the animals were killed by incising their throats, and in later experiments, by decapitation with a pair of scissors. The resultant blood of each group was collected in a beaker containing heparin. Plasma potassium values were obtained on the pooled samples by the method of Shohl and Bennet as modified by Fenn et al. (9).

Procedure. Mice were castrated at 5 weeks of age, or 12 to 18 gm. body weight. One month later they were implanted with the pellets, and at fixed intervals (table 1) 4 controls and 4 of each experimental group were killed. At autopsy the organs were removed, fixed in Bouin's fluid over night, transferred to 50% alcohol, cleaned of extraneous tissue, dried between paper towels and weighed on a Roller-Smith torsion balance.

RESULTS

Since the results of the preliminary experiments (Rockland mice) agreed very closely with the data obtained for the inbred mice (Buffalo-Marsh), only the latter are being presented. In the former, 6 groups of 4 control and 4 experimental mice were killed at 10-day intervals.

Efficacy and duration of the pellets. The growth of the accessory sex organs rapidly reaches a maximum (fig. 1). A size equal to that of the normal animal is attained within 10 to 30 days and continues with further stimulation to reach almost twice the normal size within about 40 days. It would seem, therefore, that the normal testis is not operating at a maximum capacity but at a level best suited to the physiological needs. Apparently, there is a point of maximum inherent capacity, since the responsiveness attains a steady state as exhibited by the flattening of the curves (fig. 1). It must be borne in mind that the responsiveness of the animal is influenced considerably by experimental conditions as well as inherent capacity. In previous experiments with rats (3) the maximum reaction was greatly increased by the simple expedient of administering the material at shorter intervals, thus maintaining a more uniform stimulating concentration in the blood. This effect is accomplished even better by the pellet which supplies a continuous and steady source of the hormone. Another limiting factor is the age at which castration is performed, older animals exhibiting a greater response to comparable amounts of androgen.

The rate of absorption of the pellets is such that the larger pellets would be com-

[3] The author is indebted to Professor W. O. Fenn and Miss E. Sheridan for these analyses.

TABLE 1. EFFECT OF TESTOSTERONE PROPIONATE PELLETS ON BUFFALO-MARSH MICE

Days	Pellet	Body wt.	T. prop. absorbed (total)	T. prop. absorbed per day	Sem. ves. & pros.	Sem. ves. & pros. (T. prop.)	Kidneys	Spleen	Adrenals	Liver	Ant. pit.	Thymus	Heart	Potassium	Potassium difference
		mg.	gm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	m.eq./l.	
5	Contr.	24.0	—	—	9	—	294	97	4.8	1500	1.3	73	111	11.6	—
5	22±	24.5	0.65	0.13	54	83	399	95	4.6	1550	1.3	38	125	13.9	+2.3
11	11±	24.0	1.43	0.13	104	73	350	68	3.9	1480	1.4	27	95	13.0	+1.7
13	22±	24.5	2.20	0.17	169	77	489	61	4.0	1500	1.3	13	132	13.3	+1.4
27	Norm.	25.5	—	—	189	—	393	59	3.9	1650	1.1	44	120	10.9	—
27	Contr.	24.3	—	—	9	—	207	82	5.0	1530	1.0	65	102	13.1	—
20	22±	25.8	3.75	0.19	219	58	460	78	4.3	1700	1.3	12	123	8.8	−4.3
30	Contr.	24.0	—	—	6	—	238	75	4.8	1420	0.6	53	97	9.1	—
30	22±	26.3	5.50	0.18	251	46	461	57	3.5	1570	1.1	15	108	10.9	+1.8
30	11±	26.0	3.60	0.12	196	54	512	62	5.3	1500	1.4	—	125	—	—
39	22±	25.8	7.20	0.21	239	33	495	53	4.1	1550	0.9	10	133	—	—
60	Contr.	28.3	—	—	7	—	289	84	5.6	1440	1.5	56	124	8.4	—
60	11±	26.5	7.40	0.12	266	36	601	73	5.0	1640	1.6	13	156	9.8	+1.4
60	2X11±	27.0	12.50	0.21	308	25	586	85	4.8	1860	1.6	17	146	9.8	+1.4
64	Norm.	25.5	—	—	181	—	340	55	4.0	1280	—	30	110	11.8	—
54	Contr.	26.5	—	—	4	—	242	76	5.5	1330	1.5	35	105	8.7	—
64	22±	26.3	10.90	0.17	255	23	583	62	4.5	1450	—	11	142	10.2	+1.5
90	22±	28.3	22.80	0.25	30	1	349	103	5.9	1910	—	73	131	—	—
96	Contr.	28.5	—	—	5	—	318	105	7.0	1865	—	50	121	—	—
96	22±	26.8	15.90	0.17	333	21	689	71	4.9	1940	—	15	152	—	—

pletely absorbed in 120 to 130 days, and the smaller ones in 80 to 90 days. The amount absorbed is in proportion to surface area rather than mass of the pellet. The larger pellets with the greater surface area decrease in weight at a faster rate than the smaller pellets. Furthermore, when two of the smaller pellets having a combined mass equal to and a total surface area greater than one of the large pellets are implanted, the loss in weight of these pellets is in proportion to the surface area rather than the mass (fig. 2).

In one group of animals complete absorption of the larger pellets occurred in less than 90 days (table 1). Whether this was a true absorption or a loss of the pellets

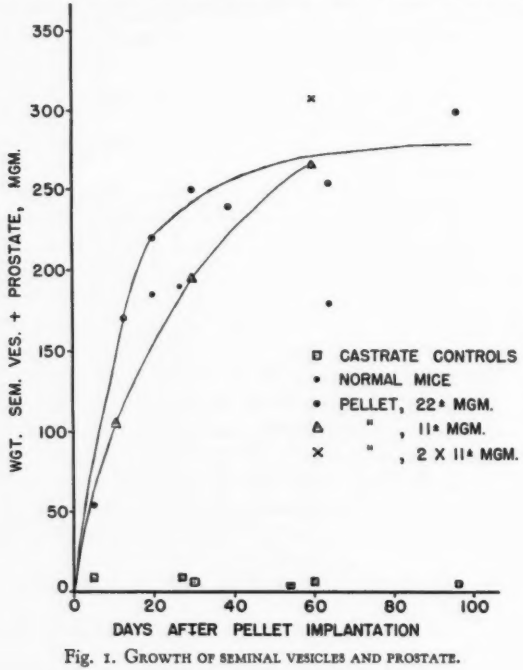

Fig. 1. GROWTH OF SEMINAL VESICLES AND PROSTATE.

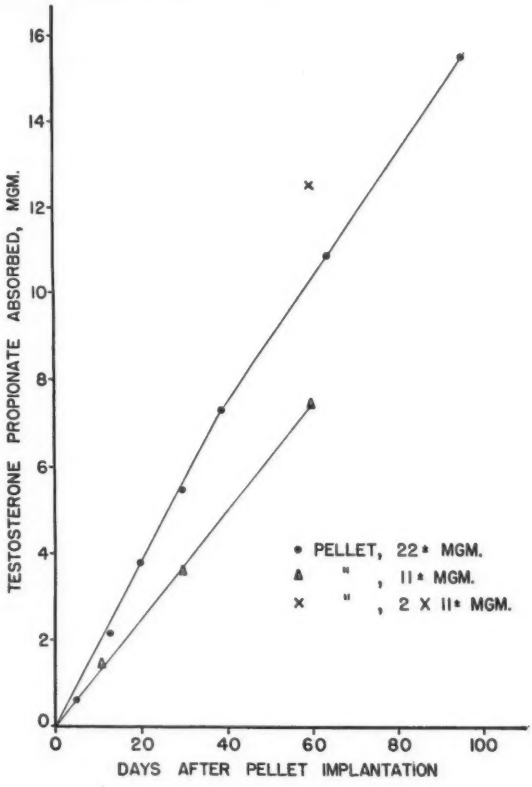

Fig. 2. Rate of absorption of testosterone propionate pellets.

through some mechanical means cannot be stated. In any event, the results are worthy of note. The loss of the influence of the pellets was of sufficient duration to return the animals toward the castrate state. The accessory sex organs had diminished to 30 mg. as compared to 5 mg. for the controls and 333 mg. for the 96-day animals. The kidneys had returned to the castrate level, the adrenals were intermediary, and the thymus had enlarged even more than that of the controls.

Kidneys. The androgen has an unquestionable effect on the size of the kidneys (fig. 4) resulting first in a substitution and later in an enlarging effect. The kidneys of the castrate animals were considerably smaller than those of the normals. This decrease was attained within the 30 days following castration, and there is no evidence of a further diminution with time. The kidney weights were not only returned to normal by the therapy but gradually increased in every experiment until in the final one (96 days) they were more than double the normal weight. No indications of gross or histo-pathology could be detected in the enlarged kidneys. In the preliminary experiments (Rockland mice) the kidneys of 50-day and 62-day treated mice and their controls were weighed fresh and then dried in an oven at 110°C. The average percentage dry weight of the 50-day controls was 23.4 and that of the experimental group, 23.3. The values for the 62-day rats were 23.5 and 22.3 respectively. There was, therefore, no indication of an accumulation of water.

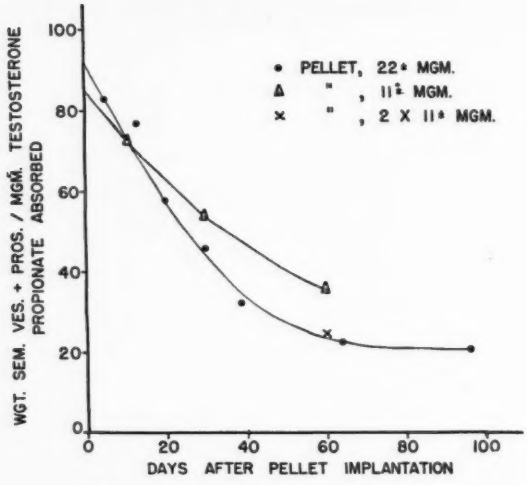

Fig. 3. Efficacy of testosterone pellets.

Thymus. The castrate control animals developed relatively large thymi, which involuted within 13 days to a minimum of 13 to 17 mg. as a result of androgen therapy. Since the thymi of the control mice were 30 to 44 mg., these organs of the experimental animals had decreased to approximately one-third as a result of the androgen therapy.

Adrenals. There is a gradual and definite increase in the size of the adrenals as a result of castration. This effect, however, does not become very noticeable until about 60 days after operation, but gradually increases until at 126 days (96 days of experiment) it has attained a value 75% greater than normal. This hypertrophy is prevented by androgen therapy.

Other organs. The heart and liver of the androgen treated mice show a small but consistent increase over that of the controls. The spleen and anterior pituitary weights were unchanged.

Potassium. The androgen treated mice with one exception show an average increase in plasma potassium of 1.64 ± 0.25 m. eq./liter over that of the castrate controls. In addition, both series of animals demonstrate a uniform parallel decrease with age.

The absolute figures for all of the experiments including two groups each of controls, experimentals and normals from the preliminary experiments gave average values of 11.35 ± 0.45 for the normals, 9.25 ± 1.79 for the castrate controls and 11.12 ± 1.17 for the treated animals. The large variability in the potassium values is due to the decrease with age. These figures nevertheless indicate that castration has produced a decrease in plasma potassium which is returned to normal by the testosterone propionate. The relatively few groups and the small difference prevent an accurate statistical analysis.

General observations. In addition to the results noted in the table and graphs, several other effects were noted. The shaved areas under which the pellets had been placed did not renew their hair even after 96 days. In general the fur of the experimental animals was shaggy, while that of the controls was sleek. These results conform with previous reports (7) obtained with the Little dba mice, in which daily in-

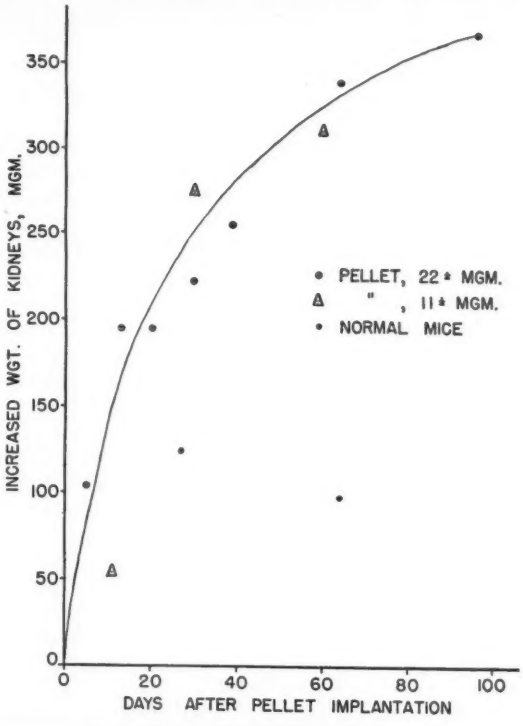

Fig. 4. INCREASE IN WEIGHT OF THE KIDNEYS.

jections of testosterone propionate produced a marked loss of hair which was more noticeable in the males; the females were only slightly affected.

The castrate controls accumulated large amounts of abdominal fat while the implanted animals had only traces.

The experimental Rockland mice were very aggressive. The normals, however, showed the same disposition. The therapy, therefore, tended to restore rather than initiate this characteristic. It is noteworthy that these aggressive tendencies were lacking in the Buffalo-Marsh mice.

There was a definite difference in the size and development of the scrota. The castrate controls showed no signs of scrotal skin, while the pellet animals had noticeably developed scrota.

The ash content of the femurs decreased as a result of castration and were brought back gradually to normal as a result of the androgen therapy. These results are to be presented in detail in another publication.

DISCUSSION

The administration of testosterone propionate pellets is an effective means of providing a continuous excess supply of the androgen to the mouse. The excess is demonstrated by three criteria: *a*) substantial increase in the size of the seminal vesicles and prostates, *b*) increase in kidney weight, and *c*) the involution of the castration-hypertrophied thymi to values below those of normal mice.

The lower effectiveness with the larger pellets indicates a greater wastage with

increased availability. No direct relationship exists between need and absorption. The animals will absorb as much as is made available, utilizing as much as they can and disposing of the excess in some other manner. It might follow, then, that in cases of excess androgen excretion there is available more than the organism requires for its normal physiology.

The effects on the organ weights are in agreement with those obtained by Korenchevsky (11) and Selye (12) for the rat and mouse (13), using injections of various androgens in oil. The effect on the kidneys is peculiarly interesting. The ability of the androgens to affect the urinary as well as the genital tract suggests at least a partial explanation for the beneficial effects noted in cases of human benign prostatic hypertrophy. The administration of androgens to these patients permits a certain amount of relief without any measurable effect on the prostate, the therapy apparently improving the tonus of the urinary tract.

It is evident that the multiple effects of testosterone propionate must be considered in the interpretation of experimental results. Unquestionably, androgen therapy produces significant physiological changes in organs that are not usually associated with the sexual mechanism.

The increased blood potassium found in the testosterone propionate treated mice conforms with the positive urinary potassium balance obtained with similarly treated human hypogonads (6). The increase, however, consists in a restoration toward the normal potassium level which has been lowered as a result of castration. It is probable that these changes are associated with the kidneys which atrophy after castration and hypertrophy after androgen therapy.

The ability of testosterone propionate to increase the plasma potassium suggests this mechanism to be responsible for the toxic effects of androgens in adrenalectomized rats (7).

SUMMARY

Rockland and Buffalo-Marsh mice were castrated at approximately 5 weeks of age. One month later, pure testosterone propionate pellets were implanted subcutaneously and the animals killed at intervals over a period of 96 days and the various organs weighed. The pellets were absorbed at a gradual rate such that the 11 ± mg. size would have been absorbed completely in 80 to 90 days and the 22 ± mg. size in 120 to 130 days. As a result of the androgen therapy, the seminal vesicles and prostates and the kidneys attained sizes greater than twice the normal, the castration-hypertrophied thymi were involuted to one-third of normal, and the castration-hypertrophied adrenals to normal, and the heart and liver were slightly enlarged. A decrease in plasma potassium as a result of castration and a return to normal by the androgen therapy was observed.

The Buffalo-Marsh mice proved very satisfactory for quantitative work.

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INHIBITION AND STIMULATION OF TESTES IN RATS TREATED WITH TESTOSTERONE PROPIONATE

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WHEN A MALE RAT, in the prepubertal period, is treated with testes extract (1) or with testosterone or testosterone propionate (2) the testes are severely damaged: they are reduced in size and weight while spermatogenesis is seriously delayed or completely arrested. When testosterone propionate was administered to adult rats, no damaging effect was observed. As early as 1932, Moore and his collaborators showed that the damage induced by male sex hormone in young rats resulted from suppression of the pituitary.

The experiments here reported were undertaken in order to study the effect of testosterone propionate treatment begun in the prepubertal period and continued beyond maturity, as well as the duration of the post treatment effect. Some observations made in the course of our experiments prompted us to include the investigation of an apparent stimulating effect of testosterone propionate.

MATERIAL AND METHODS

The inbred strain of rats used originated from the Wistar strain and was bred in our own colony for several years. The animals were fed on Purina Dog Chow with the addition of lettuce twice weekly. They were permitted free access to water. Litter-mate controls were used in every experiment. A total of 45 rats was studied. Testosterone propionate[1] was given from the first day of life to all but one series which is specifically mentioned below. Fertility was tested by mating with normal females. If no pregnancy occurred, the fertility of the females was verified by mating with normal males.

At the end of the experimental period, the animals were killed and dissected. Organ weights were taken on a torsion balance after dissecting away all connective tissue. Organs were fixed in Zenker formol, imbedded in paraffin, sectioned and stained with hematoxylin eosin. One testis was usually fixed in Susa or Bouin and imbedded in paraffin by the dioxan method, sectioned and stained as above.

RESULTS

Group 1. A. In this group each of 14 rats from 9 litters received 1 mg. of testosterone propionate 3 times weekly for periods varying from 22 to 298 days. They were killed at the end of the experiment. The weights of the testes are given in table 1. The decrease of testes weight over that of the litter-mate controls was well marked after the second month. There was no parallel between the amount of testicular weight loss and either age or total dosage. Histological examination revealed marked

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[1] We are indebted to Dr. Max Gilbert and Dr. Erwin Schwenk of Schering Corporation, Bloomfield, N. J., for the testosterone propionate used in our experiments.

arrest of maturation. At the age of 60 and 90 days, after a total dosage of 24 and 39 mg. respectively, there were no sperm heads visible. After 155 days and a total dosage of 63 mg., sperm heads were visible in some tubules of the section but no mature spermatozoa occurred. In many tubules, at varying dosage levels, there was considerable sloughing of cells and formation of giant cells. The interstitial tissue appeared reduced in quantity and the individual cells were pyknotic. It should be noted that in one animal (*L* 99) who had received as much as 57 mg. when it was killed at 85 days, the testes showed sperm heads. *Rat* 260, killed at about the same age (90 days) after having received only 39 mg., showed no sperm heads.

Group 1. *B.* Five animals were observed over a period of 8 months after discon-

TABLE 1. RATS TREATED WITH TESTOSTERONE PROPIONATE, 1 MG. 3 TIMES PER WEEK, FROM THE FIRST DAY OF LIFE

Litter No.	Total dosage	Age at death	Testes weight Treated	Testes weight Control	Increase or decrease
	mg.	*days*	*mg./100 gm. body wt.*		%
231	9	25	290	211	+13
			213	289	−17
			236	268	− 8
			258		
266	74	60	260	1218	−79
			279	1350	−79
260	39	90	209	—	
99[1]	51	86	194	752	−75
168	63	155	323	910	−65
104[1]	66	115	278	755	−64
179	65	150	281	2680	−90
148	82	192	160	650	−76
179	87	196	117	778	−84
			137		−82

[1] These animals were treated with higher doses for some time.

For calculating increase or decrease, the weight of the control testes was taken as unity. In litters with more than one control, the testes weights of the controls have been averaged and the testes of each treated animal calculated individually against the average control testes weight.

tinuing testosterone treatment. Fertility was tested in 4 of these. *Rat* 180*B* impregnated a female one and one-half months after treatment had been stopped. The animal had received a total dose of 94 mg. of testosterone propionate. *Rat* 239*T* which had received a total dose of 102 mg. was successfully mated 5 months after treatment had been discontinued. Two other rats which had received total doses of 72 mg. and 66 mg. respectively appeared to be sterile 8 months following the last testosterone injection. Mature spermatozoa were present in the testes of both the fertile and the sterile rats. However, in the sterile rats a lesser number of tubules, on cross-section, carried mature spermatozoa whereas the testicular structure of fertile animals was indistinguishable from that of normals.

Group 2. Twelve male rats were treated with 5 mg. of testosterone propionate 6 times weekly from birth for approximately 30 days. Litter-mate controls received equivalent amounts of sesame oil, the solvent for testosterone propionate. The testes of the treated animals, killed from the 25th to the 32nd day, after the animals had re-

TABLE 2. RATS TREATED WITH TESTOSTERONE PROPIONATE, 5 MG., 6 TIMES PER WEEK, FROM THE FIRST DAY OF LIFE, FOR 30 DAYS

Litter No.	Testes weight		Increase or decrease[1]
	Treated	Control	
	mg./100 gm. body wt.		%
275	650	259	+150
278	395	144	+174
281	278	178	+ 28
	556	258	+155
302	526	166	+217
	530		+219
303	374	187	+100
	417		+123
320	278	178	+ 56
321	242	139	+ 74
328	478	233	+105
	403		+ 73

[1] See footnote to table 1.

ceived 75 to 80 mg. of testosterone propionate, were heavier than those of their littermate controls (table 2). Histological examination of the testes showed the tubules to have increased in diameter. Comparison of the treated and control testes suggested stimulation of mitotic activity but the differences were not sufficiently marked to permit a definite conclusion. No sperm heads had developed in either the treated or the control animals. It should be noted also that in animals killed at 32 days (very near the normal appearance of sperm heads—36 days), no sperm heads, that is, no stimulation of maturation, were visible. No significant difference in the structure of the interstitial tissue was recognizable at this stage.

Group 3. Five rats were treated as in *group 2* for 60 days. The result was a marked decrease in testicular weight (table 3). On histological examination sperm heads were present in 3, and mature spermatozoa in one of the 5 animals each of which had received a total of 160 mg. testosterone propionate. One animal only showed a complete arrest of spermatogenesis. No sloughing of cells or formation of giant cells was found in any of the tubules in this group. The interstitial tissue showed marked damage: it was much scarcer than in the controls and the cells appeared to be pyknotic.

TABLE 3. RATS TREATED WITH TESTOSTERONE PROPIONATE, 5 MG. 6 TIMES PER WEEK FROM THE FIRST TO THE 60TH DAY OF LIFE

Litter No.	Testes weight		Increase or Decrease
	Treated	Control	
	mg./100 gm. body wt.		%
319	456	825	−45
326	227	1130	−80
	348		−70
327	494	436	+13
	276		−37

For calculation of testes weights and of increase or decrease, see note on table 1.

Group 4. In this group 9 animals were treated with 5 mg. testosterone propionate daily, 6 times weekly from the 30th to the 60th day of life. Litter mates received equivalent amounts of sesame oil. Dosage in this group was comparable to that of *group 2* while age of the animals was comparable to that of *group 3*. Of 9 males in this group, 6 had testes heavier than those of their litter-mate controls; in 3 there was no weight difference table 4. On histological examination the seminiferous tubules in the treated were larger. All the treated animals showed mature spermatozoa. The number of tubules showing complete maturation was greater in all the treated ani-

TABLE 4. RATS TREATED WITH TESTOSTERONE PROPIONATE, 5 MG. 6 TIMES PER WEEK, FROM THE 30TH TO THE 60TH DAY OF LIFE.

Litter No.	Testes weight		Increase or decrease
	Treated	Control	
	mg./100 gm. body wt.		
334	1160	755	+52
	859		+13
335	1120	655	+51
	1190	819	+61
	1190		+61
336	1420	1240	+17
	1270	1170	+ 5
	1220		+ 1
	1290		+ 7

For calculation of testes weight and of increase and decrease, see note on table 1.

mals. The interstitial tissue was scarcer in the treated than in the control testes. Pyknosis of the cells was evident.

DISCUSSION

Treatment of immature male rats with 3 mg. of testosterone propionate per week leads to marked arrest of testicular weight and size. This confirms the findings of Moore and Price (2), Schoeller and Gehrke (3), Itho and Kon (4), and Bottomley and Folley (5). Moore and Price (2) found that difference of testes weight in treated and control animals is greater in younger animals. Dosage and length of treatment were kept constant in their experiments. The experimental procedure used by us does not permit this to show up, because treatment was continued and the older animals had thereby also received a higher dosage. It is evident from our experiments, however, that continuation of treatment to the post-pubertal period does not permit the testes to regain their weight so long as treatment is continued. Nor was complete spermatogenesis seen in any of the treated animals. Sperm heads also were absent at 60 and 90 days, but were present from 150 days despite progressively increased doses of testosterone propionate.

Treatment begun in the post-pubertal period does not lead to testes damage in the rat (1, 2, 6). In adult dogs treated with various androgens and testes extracts lacking androgenic potency, Roessle and Zahler (7) found damage of the testes. Several of our animals whose treatment was begun at 100 days and who received a total dosage of 200 mg. showed normal testes histologically. But these animals proved sterile in mating experiments (which will be published at a later date). These findings are in marked contrast to the effect of treatment carried post-pubertally and begun on the first day of life, when spermatogenesis showed a marked arrest. After discon-

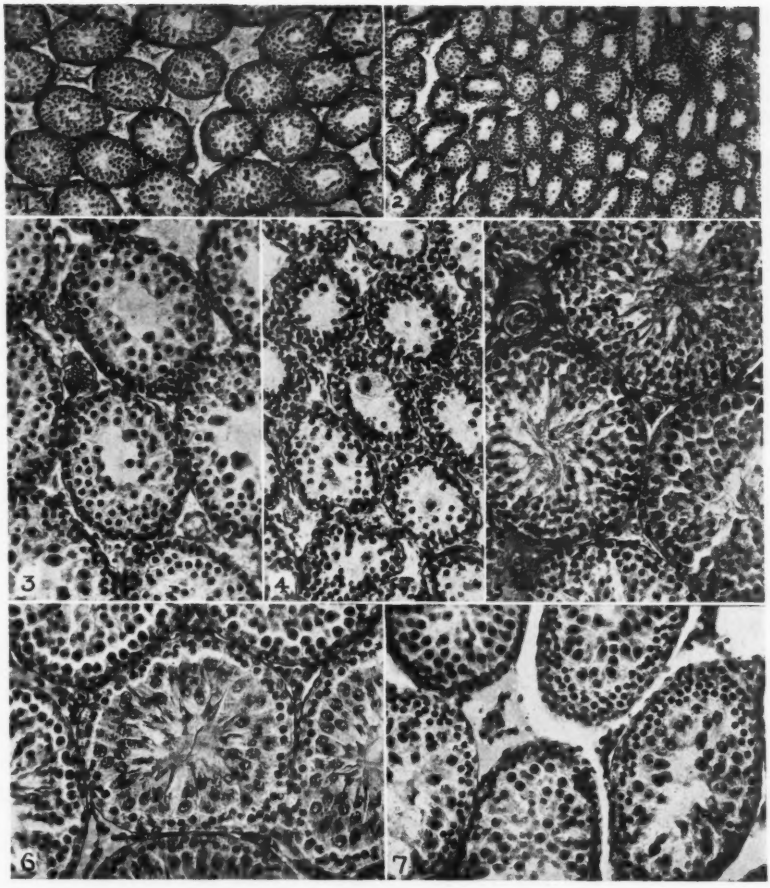

Fig. 1. *Rat* 302*B* treated with testosterone propionate for 4 weeks, beginning on the first day of life. Total dose 80 mg. Note large tubules, compare with litter-mate control figure 2. Susa, hematoxilin-eosin. ×68. Fig. 2. *Rat* 302*T*, litter-mate control animal received sesame oil. Compare with figure 1. Susa, hematoxilin-eosin. ×68. Fig. 3. Same slide as figure 1. Note large size of tubules, increased mitotic activity and damage of interstitial tissue, as compared with control (fig. 4) ×225. Fig. 4. Same slide as figure 2. Litter-mate control. Compare with figure 3. ×225. Fig. 5. *Rat* 327 *LF* treated with testosterone propionate for 60 days starting on the first day of life. Total dose 250 mg. Spermatogenesis is mature and well preserved, the interstitial tissue damaged, its nuclei pyknotic, the cells markedly shrunken. Zenker-formol, hematoxilin-eosin. ×225. Fig. 6. *Rat* 327*B*, litter-mate control treated with sesame oil. Compare with figure 5. Zenker-formol, hematoxylin-eosin. ×225. Fig. 7. *Rat* 266 *L. H.* Same age as animals in figure 5 and 6 (60 days), treated with smaller doses (3 mg. per week) Total dose 25 mg. Note complete absence of mature spermatozoa. This is the effect of testosterone propionate on the testes generally known. Compare with figure 5. Zenker-formol, hematoxylin-eosin. ×225.

tinuing treatment, normal maturation is reached in some cases at one and one-half months after the last injection of testosterone propionate and fertility can be proven. However, there are distinct individual differences which do not derive from the amount of testosterone propionate administered. Some animals remain sterile for as much as 8 months after treatment has been discontinued.

Since, after prolonged treatment with high total doses of testosterone propionate, spermatogenesis can recover completely and fertility be restored, we examined the effect of very large doses given before puberty. The surprising result was that testes weight not only did not decrease but was actually significantly increased. Moore and Price (2) have already described the paradox of large doses of testosterone propionate producing less weight loss of testes than small doses. What is the explanation?

Our results indicate how complex the problem is. The large doses continued over 2 months led to a decrease in testes weight. It is generally understood that decrease

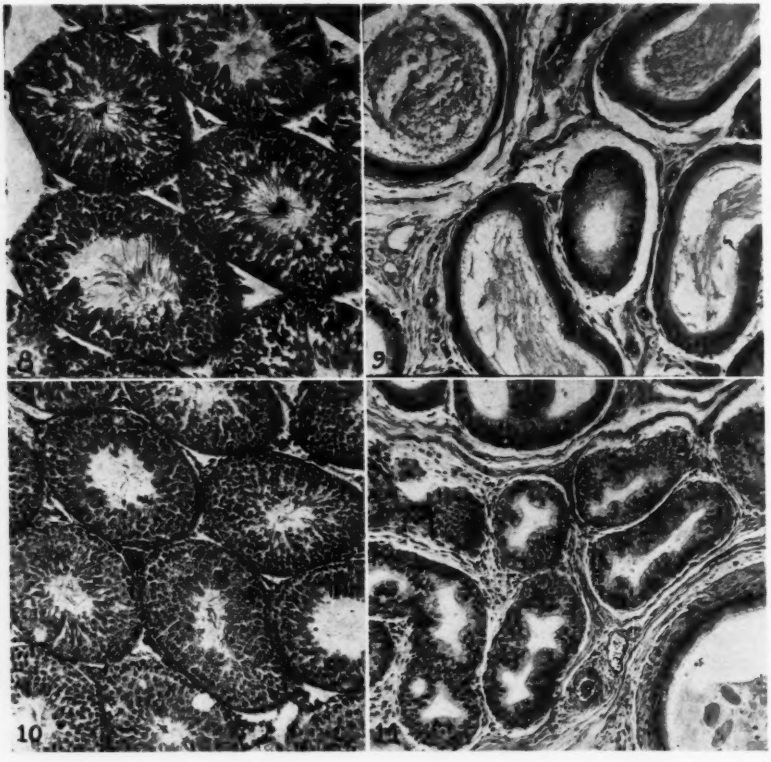

Fig. 8. *Rat 334 H.* Treated from the 30th to the 60th day of life with testosterone propionate. Total dose of 135 mg. More tubuli carrying matured spermatozoa than those of control (fig. 10). Tubuli larger. Pyknosis and shrinkage of interstitial tissue. Susa, hematoxylin-eosin. ×90. Fig. 9. Epididymis of same rat as figure 8. Some mature spermatozoa visible. Fig. 10. *Rat 334B*, litter-mate control treated with sesame oil. Compare with figure 8. Fig. 11. Epididymis of litter-mate control rat. Same as figure 10. No spermatozoa visible.

of testes weight, or more correctly arrest of testicular growth, is brought about through inhibition of the pituitary gonadotropic function. One might assume that the reaction of the pituitary is different during the first month of life; namely, reacting to large doses of testosterone propionate with an increased output of gonadotropic hormone. In the second months, however, the pituitary reaction would be that of inhibition. But this cannot be the case, because giving testosterone propionate from

the 30th to the 60th day of life leads to an increase in testes weight. Moreover, large doses of testosterone propionate, as employed in our experiments, influences not only testicular weight differently from smaller doses, but spermatogenesis as well. In the first month of life spermatogenesis would appear to be increased in the treated animals as compared with their litter-mate controls, inasmuch as mitotic activity seems to be increased. No hastening of maturation occurs, however, and none of the animals reached the sperm head stage at 32 days. More conspicuous is the influence on spermatogenesis when testosterone propionate is given from the 30th to the 60th day. Then the testes carry mature spermatozoa which is in marked contrast to the results obtained in animals of this age with smaller doses which lead not only to an arrest of testicular weight but also a complete arrest of maturation of spermatozoa.

There are definitely more tubules carrying mature spermatozoa than in the corresponding litter-mate controls. Whereas testosterone propionate does not hasten maturation in the sense that mature spermatozoa or sperm heads are produced at an earlier age, one gains the impression that in the dosage employed in our experiments, it does enhance maturation once the intrinsic possibility for maturation is reached.

In *group* 3, in the animals that received a total dosage of 225 mg. within the first 2 months of life, sperm heads were present in 3 and some mature spermatozoa in a fourth of the 5 treated animals. Only one showed a complete arrest of maturation. In comparison, the testes of a litter that had received 25 mg. within the first 2 months of life showed complete absence of sperm heads or mature spermatozoa. Treatment with large doses over a period of 2 months inhibits maturation to a lesser degree than smaller doses, even though growth of the testes is inhibited.

If these changes should be due entirely to depression or stimulation of the output of gonadotropic hormone from the pituitary the following assumption would have to be made: small doses of testosterone propionate inhibit the gonadotropic output, large doses stimulate the output, but continuing large doses, over a longer period thus increasing the total amount administered, in turn decreases the gonadotropic output. Aside from the improbability of such an action, there are facts which are at variance with this assumption. A considerable increase in gonadotropic output would be expected to hasten testicular descent. This, however, is not the case. Descent of the testicles occurs at the same time in both treated and control animals. Furthermore, stimulation of output of gonadotropic hormone should be recognized in the ovaries of the females treated in the same way as the males. This again is not the case. Female members of the litters used in these experiments were treated in the same way as the males. Even though this report deals exclusively with the males, we wish to state that the ovaries in *groups* 3 and 4 show atrophy and maturation arrest. Unless one wishes to assume that testosterone propionate in large doses stimulates the pituitary of the males only and inhibits the pituitary of the females, the findings in the females do not favor the assumption of pituitary stimulation by large doses of testosterone propionate. Furthermore, pituitary inhibition becomes evident even in the large and stimulated testes from the damage visible in the interstitial tissue.

Several authors have reported a stimulating effect of testosterone propionate on the testes. Rubinstein (8), Rubinstein and Kurland (9) have found an increase of spermatozoa count of men treated with testosterone propionate. Increase of dosage conversely led to a decrease of spermatozoa. Motility also was improved by testosterone propionate treatment. Other authors (10, 11, 12) saw oligospermia occur in the course of testosterone treatment. Differences of dosage as well as differences of susceptibility of the treated individuals seem to account for the variance of results. Wells and Moore (13) found that androgens produced precocious spermatogenesis in the ground squirrel. Rubinstein (8) has reported a certain amount of stimulation of

the germinal epithelium in testosterone treated immature rats. Testosterone propionate as well as other androgens are capable of maintaining spermatogenesis in the hypophysectomized rat (14, 15). Even though some doubts as to the specificity of this action have been voiced (16), a dual action of testosterone propionate would offer a tentative explanation for our results. Testosterone propionate could then inhibit the gonadotropic output for the pituitary and at the same time stimulate the testes. These effects are then antagonistic: pituitary inhibition leading to arrest of testicular growth and of sperm maturation; testicular stimulation enhancing growth and maturation. The result of an individual experiment would depend upon the predominance of either action.

During the first 30 days the direct stimulating action of the large doses of testosterone propionate would prevail over pituitary inhibition. During a further month of continued large doses, pituitary inhibition would become marked with arrest of testicular growth and sperm maturation. Stimulation, however, is not completely masked because inhibition of sperm maturation is incomplete: there are sperm heads and in a single instance mature spermatozoa. If, on the other hand, treatment with large doses is begun only on the 30th day of life, the stimulating effect prevails.

The absence of signs of pituitary gonadotropic inhibition in the early age group (1–30 days) may be explained by the fact that the pituitary of the male rat shows no gonadotropic activity up to the 13th day of life (17) with a gradual increase of gonadotropic activity reaching its peak between 27 and 30 days (18). At this age, the animals in our series were killed; that is, they had received treatment only to a time when relatively little, or no, gonadotropic pituitary function existed. Absence of damage to the interstitial tissue in this age group would also lend support to such a view.

In the group treated from the 30th to the 60th day of life, treatment was given during a period when the pituitary gonadotropic activity had reached its peak. The inhibition of the latter is shown by the damage to the interstitial tissue. The stimulating direct effect of large doses of testosterone propionate on the germinal tissue still prevailed in this group.

Smaller doses such as are ordinarily employed inhibit the pituitary and consequently testes weight and structure are impaired. We have to assume then that the smaller dosage of testosterone is not sufficient to stimulate the testes directly but that it is sufficient to suppress pituitary action. Large doses also suppress the pituitary, but the direct stimulating action on the testes contributes to the final outcome. If the large doses are given during two months pituitary inhibition is so pronounced that the final result is intermediate; partial impairment of the testes, yet less than with small doses because of the counteracting direct stimulation.

Nelson and Merckel (15) showed that testosterone, the most potent androgen, is the weakest of all androgens examined in maintaining spermatogenesis of the hypophysectomized rat (with the exception of transandrostenediol which is still weaker). This may explain why stimulation is seen only with large doses of testosterone propionate whereas the inhibitory influence on the pituitary is exerted by much smaller doses. Only the youngest age group treated from birth to the 30th day failed to show damage to the interstitial tissue. This group was discussed above. It seems probable that the damage to interstitial tissue is due to pituitary gonadotropic inhibition though at the present time opinion as to the nature of the action of the pituitary on the interstitial tissue varies. According to Greep, Fevold, and Hisaw (19, 20), the FSH factor maintains gametogenesis, the LH factor the interstitial tissue. Zeckwer (21) recently published experiments which would indicate that the castrate pituitary secreting predominantly FSH stimulates the interstitial cells and not the germinal epithelium. However, the latter fact might be due to the use of adult animals. Moore

and Price found that the interstitial tissue reacts more extensively to various gonadotropins than the germinal epithelium. Rubinstein and Abarbanel (22) found that sheep's pituitary extract stimulates both the germinal cells and the interstitial tissue. It seems of interest, in view of our findings, that androgens should be capable of maintaining spermatogenesis in the hypophysectomized rats but failed to maintain the interstitial tissue (14).

Our experimental results in the intact animal revealed the same; testosterone propionate seems to act directly on the germinal epithelium only, whereas the interstitial tissue is damaged. This damage would indicate, in our opinion, the inhibition of the gonadotropic output of the pituitary. Whether the inhibition of germinal epithelium as well as decrease in testes size are dependant upon pituitary inhibition on both interstitial tissue and germinal epithelium or whether the pituitary inhibition leads to damage to interstitial tissue followed by impaired spermatogenesis, cannot be decided at the present monent.[2]

SUMMARY

Treatment of young rats with 3 mg. of testosterone propionate per week leads to decrease of testes weight, inhibition of sperm maturation, and damage to the interstitial tissue. These inhibitory effects are present also when treatment is carried on in the postpubertal period, whereas treatment begun in the postpubertal period does not produce such an inhibition. After treatment is discontinued, spermatogenesis is partially restored and some of the animals become fertile.

Treatment of young rats with very much larger doses, 30 mg. per week, results in increase in testes weight and stimulation of spermatogenesis both in rats treated from the first to the thirtieth day and in those treated from the thirtieth to the sixtieth day of life. When treatment with the large doses (30 mg. per week) is continued from the first to the sixtieth day of life, testes weight is decreased and spermatogenesis is inhibited. This inhibition, however, is less marked than it is in rats of the same age treated with 3 mg. per week. The interstitial tissue shows signs of damage in both the animals with stimulation and in those with inhibition of testes growth and spermatogenesis.

Stimulation of spermatogenesis is possible only within limits. The immature germinal epithelium can be stimulated by testosterone propionate to increased activity but not to an earlier maturation. Once a stage is reached at which maturation intrinsically becomes possible, testosterone propionate may stimulate maturation quantitatively. The treated animals' testes then carry more mature spermatozoa than those of the controls.

As a tentative explanation, testosterone propionate is thought to stimulate the testes directly, at the same time exerting an inhibitory influence on the pituitary. This in turn inhibits the testes through inhibition of the gonadotropic pituitary hormone. During the first month of life the stimulating effect is not counteracted by inhibition of the pituitary, because gonadotropic function is not yet developed. In later life, the effect on the testes is intermediate between direct stimulation and indirect inhibition. Very large doses are necessary to make the stimulating effect dominant, leading to the assumption that the pituitary gonadotropic inhibition is brought about by doses smaller than those needed for direct stimulation of the testes; that is, the pituitary is more sensitive to testosterone action than the testes.

We gratefully acknowledge the technical assistance of Miss Miriam P. Blum.

[2] It should be briefly noted that the scrotum was not visibly impaired in any of the groups. Spermatogenesis is known to be dependant on a normal scrotum, but the scrotal factor does not seem responsible for the difference of testicular weight and structure in our experiment.

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THE EFFECT OF TESTOSTERONE PROPIONATE ON THE RAT TESTIS[1]

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CURRENT REPORTS dealing with the effect of testosterone propionate on the testis of the rat are confusing. Moore and Price (1) injecting respective groups of males daily from 20 to 39 days with 50γ, 0.5 mg. and 1 mg. found evidence of true tissue damage in the testes of each group and concluded that the harmful effects decreased as the hormone dosage increased. Korenchevsky and Hall (2) injecting two groups of senile rats (14 to 18 months of age) with doses of 0.75 mg. and 7.5 mg. respectively daily for 24 days observed depression of the testis which was approximately similar in both groups. In younger adults (final ages 137–174 days) the same amounts injected 5 times weekly for 90 to 118 days again led to depression but here, as in the report of Moore and Price, the smaller dosage seemed to be more deleterious. In a third series of experiments Korenchevsky, Dennison and Hall (3) treated two groups of animals with daily doses of 0.5 mg. and 1.5 mg. respectively from 80 to 102 days of age. They noted that testicular weights were depressed to an approximately similar degree (a difference of 5% in weight between the two groups) but that histologically the testes of all animals were normal and spermatogenesis was progressing similarly in both treated and untreated groups.

Biddolph (4) working with smaller doses, administered 2γ and 10γ to respective groups beginning injections at the first day of life. After 31 days of treatment the testes of the animals receiving 10γ weighed one eleventh (1/11) of those of the controls. Continuing the treatment with the same daily dosage until the rats were 80 days of age, however, resulted in testes which showed only slight difference from their controls both in weight and misroscopically. Those animals treated with 2γ daily and killed at 30, 36, 40 and 44 days respectively showed only slight inhibition of growth but no histological abnormalities.

While some of these reports indicate that testicular damage fails to occur with certain small doses of testosterone propionate they all agree that inhibition of testicular growth in the young and perhaps depression of testicular weight in the adult occurred with all doses used.

Since testosterone propionate is now being used clinically in adolescent and adult males it is very important to determine whether or not all effective doses of this hormone will result in testicular damage. 'Effective dosage' is stressed since it is conceivable that an infinitesimal dose may spare the testis but at the same time be too small to exhibit any androgenic properties.

For this purpose a total of 150 male albino rats (*Mus norvegicus* var. *albus*) of Wistar Institute strain were studied. These were divided in 3 groups which were

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then subdivided and treated as shown in table 1. The animals receiving short term treatment were injected either intraperitoneally (*group 1*) or subcutaneously (*group 2*) for 10 consecutive days with testosterone propionate (perandren) beginning at 21 days of age. In the chronic experiments injections were given intraperitoneally daily (except Sunday) from 26 to and including 79 days of age. All treated animals and their respective litter-mate brother controls were killed 24 hours after the last injection. Except for the injections to the test animals, treatment was similar for all groups. Purina Dog Chow and green vegetables were used as food, and water was constantly present. The controls were not injected because it was found from previous studies (5) that the injection of inert substances failed to influence the growth curve. In addition, the sesame oil used as a solvent in these experiments is sexually non-stimulating (6).

Body weight was taken before and after the treatment period. All animals were killed by sectioning the carotids while under ether anesthesia. In groups 1 and 3, tes-

TABLE 1. THE SUBDIVISION OF ANIMALS, THE DOSAGE OF TESTOSTERONE PROPIONATE, THE DURATION OF TREATMENT AND THE MODE OF ADMINISTRATION IN EACH GROUP

		20 non-treated controls	
Group 1	10 received	20 γ	Intraperitoneally daily for 10 days beginning at 22 days of age. Killed at 32 days.
50 animals	10 received	1.0 mg.	
	10 received	2.5 mg.	
		20 non-treated controls	
Group 2	10 received	5 γ	Sub-cutaneously daily for 10 days beginning at 22 days of age. Killed at 32 days.
	16 received	10 γ	
66 animals	10 received	20 γ	
	10 received	50 γ	
		12 non-treated controls	
Group 3	12 received	50 γ	Intraperitoneally daily (except Sunday) beginning at 26 days of age. Killed at 80 days.
34 animals	10 received	2.5 mg.	

tes and epididymides, penis, prostate and seminal vesicles including the coagulating glands were excised, weighed, fixed in Bouin's solution and stained by hematoxylin and eosin for microscopic study. In *group 2* only testes and seminal vesicles were excised and studied. The data obtained were massed for each group and analyzed statistically by Fisher's (7) method designed for small series. The results were then tabulated in tables 2 to 4. Differences between treated and control figures were expressed in percentage. This procedure permits a clearer appreciation of the magintude of deviation and discloses more strikingly the relative response of the different tissues.

In this study Fisher's t had to be large enough to lead to a P (probability) equal to or less than 0.05 in order for observed differences to be considered as 'probably significant.'' It so happened, however, that whenever a difference was observed to be 'probably significant,' P was always less than 0.01, indicating a still higher probability of significance.

RESULTS

From table 2 it is seen that neither initial nor final body weights differed materially between the treated and control groups. The seminal vesicles, prostates and penes were significantly increased in all treated animals. The testes and epididymides were depressed in all groups.

From table 3 it may be seen that body weight again remained unaffected by all dosages in the short term experiments while the seminal vesicles became significantly

TABLE 2. THE EFFECT OF TESTOSTERONE PROPIONATE GIVEN INTRAPERITONEALLY IN DOSES OF 20 γ, 1 MG. AND 2.5 MG. DAILY FOR 10 DAYS (22–32 DAYS OF AGE) TO RESPECTIVE GROUPS OF MALE ALBINO RATS

	Control	20 γ group		1 mg. group		2.5 mg. group	
	Wt.	Wt.	% diff.	Wt.	% diff.	Wt.	% diff.
Testes and epididymis (mg.)	612.6	455.6	−26 s[1]	473.4	− 23 s	508.3	− 17 s
Penis (mg.)	50.1	77.8	+55 s	108.4	+116 s	105.1	+110 s
Prostate (mg.)	61.3	85.2	+39 s	182.2	+197 s	264.4	+332 s
Seminal vesicles (mg.)	21.8	38.9	+78 s	199.8	+818 s	178.6	+718 s
Initial body wt. (gm.)	32.1	31.8	− 0.9 i	34.5	+ 7.5 i	29.0	− 10.3 i
Final body wt. (gm.)	60.8	66.4	+ 8.0 i	66.2	+ 9 i	55.6	− 9.3 i
No. of animals	20	10		10		10	

[1] The significance or insignificance of difference is indicated by s or i respectively.

enlarged in all treated groups. With 50γ and 20γ daily the testes became significantly depressed while with 10γ dosage the weight of the testes remained practically unchanged. The testes of animals treated with 5γ, however, became significantly heavier than the control testes.

TABLE 3. THE EFFECT OF TESTOSTERONE PROPIONATE GIVEN SUBCUTANEOUSLY IN DOSES OF 5, 10, 20 AND 50γ DAILY FOR 10 DAYS (22–32 OF AGE) TO RESPECTIVE GROUPS OF MALE ALBINO RATS

	Control	5 γ group		10 γ group		20 γ group		50 γ group	
	Wt.	Wt.	% diff.	Wt.	% diff.	Wt.	% diff.	Wt.	% diff.
Testes (mg.)	563.0	659.0	+17 s[1]	546.0	− 3 i	450.0	−20 s	276.0	− 51 s
Seminal vesicles (mg.)	22.5	29.0	+29 s	33.5	+49 s	37.0	+64 s	52.0	+131 s
Initial body wt. (gm.)	30.9	33.3	+ 7.8 i	32.1	+ 3.9 i	29.1	− 5.8 i	30.1	− 2.6 i
Final body wt. (gm.)	70.8	75.3	+ 6.4 i	73.8	+ 4.2 i	67.8	− 4.2 i	67.8	− 4.2 i
No. of animals	20	10		16		10		10	

[1] The significance or insignificance of differences is indicated by s or i respectively.

From table 4 which summarizes the results obtained in the long term experiments it may be observed that animals treated with 50γ daily showed a significant gain in weight while the 2.5 mg. group failed to show this growth stimulation effect. The seminal vesicles, prostates and penes were significantly heavier than those of the controls in both groups. The testes of the 2.5 mg. group were significantly depressed but testicular weight in the 50γ group appeared to be unaffected.

TABLE 4. THE EFFECT OF TESTOSTERONE PROPIONATE GIVEN INTRAPERITONEALLY IN DOSES OF 50 γ AND 2.5 MG. DAILY (EXCEPT SUNDAY) FROM 26 TO 80 DAYS OF AGE TO RESPECTIVE GROUPS OF MALE ALBINO RATS

	Control	50 γ group		2.5 mg. group	
	Wt.	Wt.	% diff.	Wt.	% diff.
Testes and epididymis (gm.)	2.88	2.93	+ 1.7 i[1]	2.06	− 29 s
Penis (mg.)	188.7	223.1	+18 s	229.0	+ 21 s
Prostate (mg.)	315.9	528.3	+67 s	649.3	+105 s
Seminal vesicles (mg.)	414.8	536.6	+29 s	743.7	+ 79 s
Initial body wt. (gm.)	33.1	36.3	+ 9.6 i	36.8	+ 11 i
Final body wt. (gm.)	167.9	203.2	+21 s	185.0	+10 i
Gain in body wt. (gm.)	134.8	163.8	+22 s	138.5	+ 2.7 i
No. of animals	12	12		10	

[1] The significance or insignificance of differences is indicated by s or i respectively.

Microscopically, it was observed that the testes of respective groups treated with either 5, 10, 20, 50 or 1000γ daily for 10 days presented essentially normal pictures in spite of their difference in weight response. The testes of animals receiving 2.5 mg. for 10 days, however, did already show definite retrogressive features as indicated by the diminution in tubular diameter and their poor staining qualities.

The epididymides of many of these treated animals regardless of dosage, contained numerous actively mitotic immature spermatozoa. The normal epididymis only occasionally contained such testicular products (fig. 1).

Of the animals treated from 26 to 80 days, those receiving 50γ daily (except Sunday) showed no essential testicular change from the normal. The animals treated with 2.5 mg. daily (except Sunday), however, possessed testes displaying marked evidence of degeneration and spermatogenetic inhibition. The tubules appeared shrunken, the cellular architecture became dulled and spermatozoa were almost entirely absent (fig. 2–4).

DISCUSSION

From the foregoing results it is seen that all doses of testosterone propionate used were androgenically potent as judged by their stimulating effect on the prostate, seminal vesicles and penis. Body weight was not affected in any group within 10 days but the animals treated with 50γ over the longer period did show a gain which was 22% in excess of the gain shown by the controls. This stands in contrast to the growth depressing effect observed in the castrate male rat (8) and will be considered in detail elsewhere (9). From previous studies (10) one would expect that the extended use of such large doses as 2.5 mg. daily would depress the growth of the animal and yet the weight of these animals remained apparently unaffected. The reason for this discrepancy is not known but it is possible that after the induction of a maximum depression, in pituitary growth effect by large doses of testosterone propionate, the administration of still larger doses stimulates body growth directly thereby counteracting the pituitary growth depression. This problem will also bear further experimentation.

The degree of response to testosterone propionate seems to differ with each sex organ. This may best be seen from figure 5 which represents the effects of the different doses used. In the younger (10-day) group both penes and seminal vesicles reached their maximum sizes with 1 mg. doses. While for the penis this represented an approximate doubling of size (116% increase) for the seminal vesicles it meant an approximate 8-time increase (818%). In the 80-day groups doses of 50γ and 2.5 mg. led to approximately similar penile response (18% and 21%, respectively) but the seminal vesicles of the 2.5 mg. group became much heavier (79% increase) than those of the animals treated with 50γ (29% increase).

The prostate glands of both 10-day and 80-day groups continued to increase in size with progressively larger doses apparently indicating that these organs had not reached their maximum growth capacity with any of the dosages used. It seems that when an organ once reaches a maximum weight, further increase in dosage tends towards depression (fig. 5).

It is difficult to see from these data how the prostate can be considered to have a lower threshold than the seminal vesicles as advocated by Moore and Price (1). It is true that after long term treatments the prostates had shown a higher percentage increase than the seminal vesicles but in the younger animals the reverse was true, i.e. the seminal vesicles were always heavier, thus indicating an initial higher degree of sensitivity.

It is interesting to observe that similar doses had a less stimulating effect on the 80-day animals than on those killed at 32 days. The most striking example of this phe-

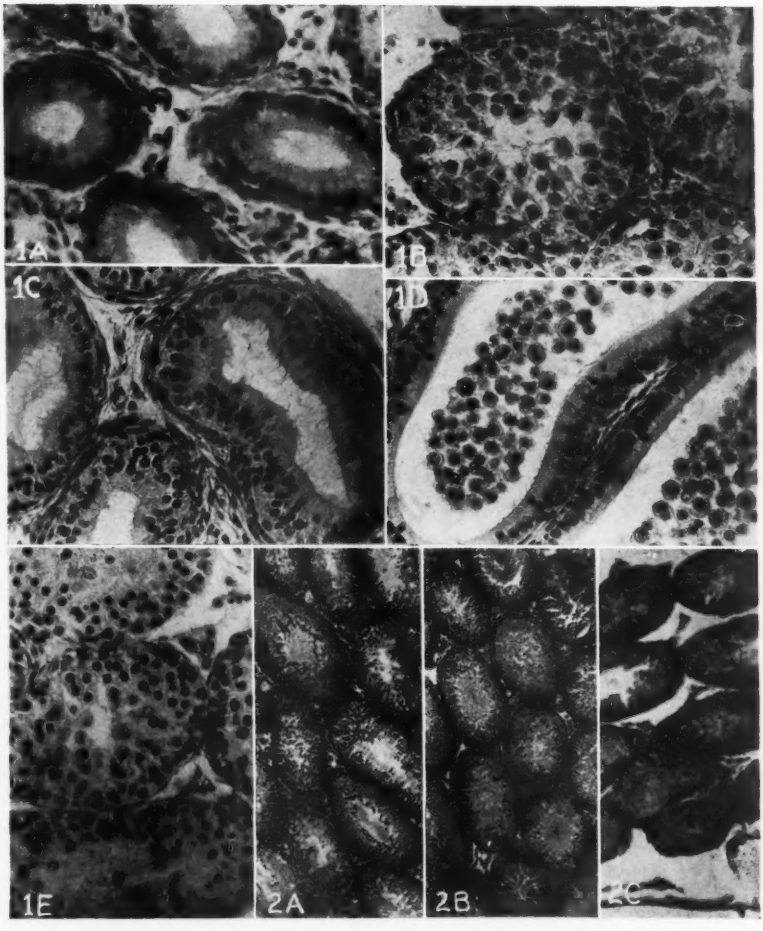

Fig. 1. Testes and epididymides of 32-day-old albino rats (×60). A, control epididymis; B, control testis; C, epididymis of animal receiving 20γ testosterone propionate intraperitoneally daily (except Sunday) from 22–32 days. Tubular wall appears thickened; D, Same as C showing the presence of actively mitotic premature spermatozoa in the tubules; E, testis to D showing essentially normal picture.

Fig. 2. Testes of 80-day-old albino rat (×65). A, Control; B, From animal receiving 50γ testosterone propionate daily (except Sunday) from 26–80 days showing no essential change from the normal; C, From animal receiving 2.5 mg. testosterone propionate daily (except Sunday) from 26–80 days showing absence of spermatozoa and tubular shrinkage.

nomenon is seen in the effect upon the seminal vesicles which showed an 818% increase with 50γ given to young animals for 10 days and only a 29% increase when given daily (except Sunday) to animals from 26 to 80 days of age. This is in keeping with the observations resulting from Biddolph's (1) studies with testosterone propionate and Moore's (11) report on gonadotropic substances.

The response of the testes to the treatment is extremely engaging since depending upon the dosage used testicular weight was either diminished, uninfluenced or

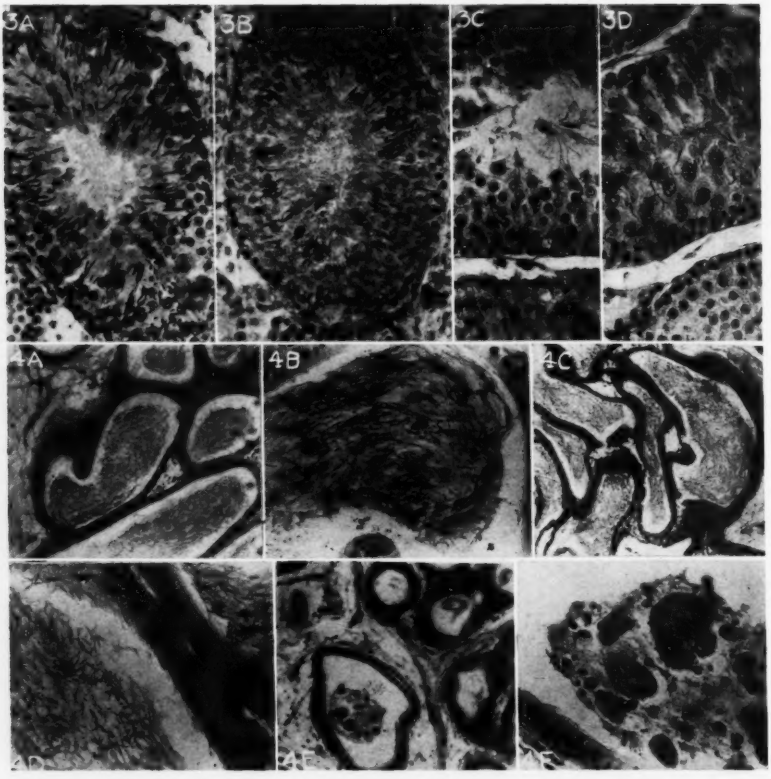

Fig. 3. Testes of rat at 80 days of age (×200). A, Control showing abundance of fully developed spermatozoa; B, From animal treated with 50γ testosterone propionate intraperitoneally daily (except Sunday) from 26–80 days of age, essentially normal picture; C, and D, From animals treated with 2.5 mg. testosterone propionate intraperitoneally daily (except Sunday) from 26–80 days showing paucity of mature spermatozoa.

Fig. 4. Epididymides of 80-day-old albino rats. A, Control showing testicular products in tubules (×50); B, Same as A showing spermatozoa in tubules (×200); C, From rat receiving 50γ testosterone propionate intraperitoneally daily (except Sunday) from 26–80 days showing essentially normal picture; D, Same as C showing spermatozoa in tubule (×200); E, From rat receiving 2.5 mg. testosterone propionate intraperitoneally daily (except Sunday) showing abnormal concretions in the tubules; F, Same as E showing sloughed-off immature sperm cells mingled with unorganized debris (×200).

increased. In the 10-day experiments the testes reached their maximum depression with 50γ. All doses larger than 50γ depressed testicular weight but the depression was progressively less the larger the dosage used (fig. 5). This substantiates the reports of those who have hitherto observed less testicular depression with increased dosage (1, 2). These investigators, however, did not use less than 50γ daily.

With quantities progressively less than 50γ the testis responded with progressively less depression so that with 10γ no significant deviation could be detected and with 5γ the testis actually increased in size. This seems to be the first time that testosterone propionate has been observed to stimulate testicular growth. It is true that Biddolph (1) also reported a negligible depression with his small (2γ) doses adminis-

tered from birth but his report failed to show that the hormone at the dosage used was androgenically potent.

In our older group of animals 50γ doses resulted in essentially unaltered testes (fig. 5) in spite of the fact that this same dosage given for only 10 days at an earlier age led to the maximum depression. This observation is to some extent in keeping with Biddolph's report concerning the relatively benign effect of continued use of 10γ after a short-term trial had resulted in a 91% weight decrease.

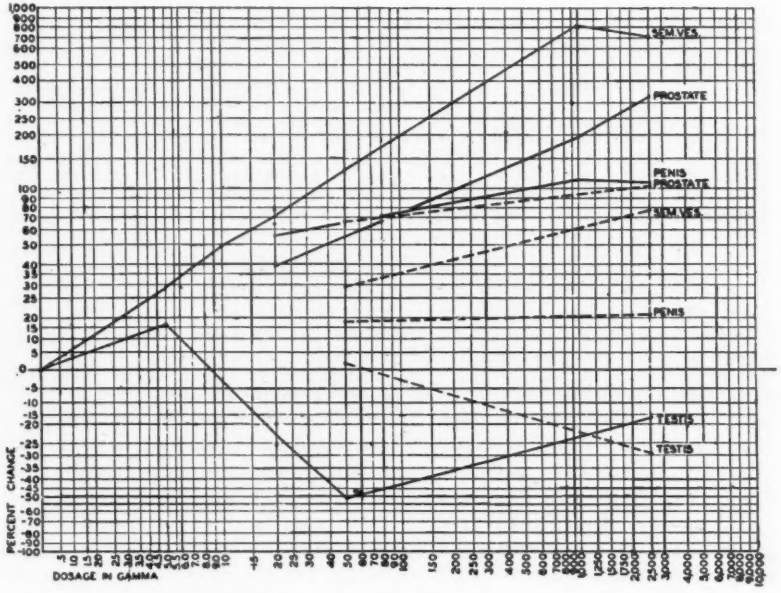

Fig. 5. THE RESPONSE OF VARIOUS SEX ORGANS TO DIFFERENT DOSES OF TESTOSTERONE PROPIONATE. Solid lines indicate 10-day experiments. Broken lines indicate 54-day experiments.

It seems, therefore, that the response of the testis to continued treatment with testosterone propionate depends among other things upon 4 factors exerting their respective influences simultaneously. These are: the size of the dose; the duration of treatment; the age (body weight) of the animal; the secondary sex organ response.

From table 5 it may be seen that, when calculated on the basis of dosage per gm. of body weight, similar daily doses when given through different age periods represent varying concentrations. For example, 10γ daily given from birth to 31 days represents 0.6γ per gm. per day while the same dosage extended through 81 days because of the progressive increase in body weight dwindles to 0.2γ per gm. per day. In the one case spermatogenesis and weight are inhibited while in the other, they remain normal. Having the body weights of our animals at our disposal it was possible to construct table 6 for our series on the basis of actual rather than theoretical body weights. Comparing tables 5 and 6 shows how originally similar doses given for different durations of time affect the organism in different concentrations.

Table 6 also shows that the degree of testicular degeneration is not necessarily related to the degree of weight depression. For example, the 2.5 mg. (for 10 days)

TABLE 5. RESULTS OF SEVERAL AUTHORS ON THE EFFECT OF VARYING DOSAGES OF TESTOSTERONE PROPIONATE GIVEN AT DIFFERENT AGE PERIODS ON TESTICULAR WEIGHT AND HISTOLOGY

	Age period of treatment	Duration of treatment	Total dosage		Dosage per gm. body wt.[1]		Effect on testis	
			Per day	Through exp't	Per day	Through exp't	Wt.	Histology
	days	*days*						
Author	1–31	30	10	300	0.60	18.0	Diminished to 1/11	Spermatogenesis inhibited
Biddolph	1–51	50	10	500	0.37	18.5	Less diminished	Less inhibition
	1–81	80	10	800	0.20	16.0	Slight decrease	Few abnormalities
	1–31	30	2	60	0.12	3.6	Very little	No abnormalities
	1–36	35	2	70	0.10	3.5	Diminution	
	1–40	39	2	78	0.09	3.5	No statistical analysis	
	1–44	43	2	86	0.09	3.9		
Korenchevsky	80–101	21	500	10,500	3.10	65.1	Decreased 15%	No abnormalities
	80–101	21	1500	31,500	9.30	195.3	Decreased 10%	No abnormalities
Moore and Price	20–40	20	50	1,000	1.7	34.0	Decreased 72%	Degeneration
	20–40	20	100	2,000	3.4	68.0	Decreased 75%	Degeneration
	20–40	20	500	10,000	17.0	340.0	Decreased 40%	Degeneration
	20–40	20	1000	20,000	34.0	680.0	Decreased 19%	Degeneration
	70–90	20	2000	40,000	15.0	300.0	Decreased 1–10%	Not mentioned

[1] The dosages per gm. body weight were calculated on the basis of mean theoretical body weights through the duration of treatment as given by Donaldson.

testes which had become only 17% less in weight showed a markedly degenerative picture (fig. 2) while the 1 mg. (for 10 days) testes which had become 22% lighter than the controls appeared to be essentially normal microscopically. This suggests the possibility that spermatogenesis may be inhibited by certain doses without the onset of testicular degeneration. As a matter of fact, from the contents of the tubules of the epididymides it appears that testosterone propionate like a number of gonadotropic hormones (12, 13, 14) whips up the proliferation and desquamation of the germinal epithelium without, however, hastening maturation. In this respect it may be said to produce what may be considered as a testicular catharsis which results in the premature elimination of spermatozoal elements. The effect of testosterone propionate

TABLE 6. THE EFFECT OF VARIOUS DOSES OF TESTOSTERONE PROPIONATE ON TESTICULAR WEIGHT AND HISTOLOGY

Group	Age period	Duration	Total dosage		Dosage per gm. body wt.		Effect on testes	
			Per day	Through exp't	Per day	Through exp't	Wt.	Histology
	days	*days*	γ	γ	γ	γ		
1	26–80	54	2,500	135,000	22.50	1,215	Sig. dec. (28%)	Degen. and inhib. of sperm.
2	26–80	54	50	2,700	0.42	22.7	No change	No abnormalities
3	22–32	10	2,500	25,000	59.00	590.0	Sig. dec. (17%)	Degenerative phenomena
4	22–32	10	1,000	10,000	20.00	200.0	Sig. dec. (22%)	Essentially normal
5	22–32	10	50	500	1.00	10.0	Sig. dec. (51%)	Essentially normal
6	22–32	10	20	200	.40	4.0	Sig. dec. (20%)	Essentially normal
7	22–32	10	20	200	.40	4.0	Sig. dec. (26%)	Essentially normal
8	22–32	10	10	100	.18	1.8	No change	Essentially normal
9	22–32	10	5	50	.09	0.9	Sig. inc. (17%)	Essentially normal

differs from the gonadotropic hormones, however, in that it fails to stimulate the interstitial tissue.

It appears from our studies that the further response of respective groups to continued treatment with different dosages is related, among other things, to the response already shown by the accessory organs to previous treatment. This position seems to be a compromise between the attitude of Hertz and Meyer (15) who disclaimed any such relationship after treating animals for only 5 days and the report of Jacobsen and Christensen (16) who held the reacting tissue to be the one and only factor influencing such response.

In our own studies it was observed that the accessory sex organs generally became progressively heavier the larger the dose of hormone used. It is conceivable, therefore, that after such enlargement, the hormone subsequently injected was used for the most part to maintain these giganticized accessories.

What constitutes a large or small dose also requires comment. As already mentioned Moore and Price considered 50γ of testosterone propionate to be a small dose. In the recent report of Green and Burrill (17) showing the recuperative capacity of testes after previous depression with testosterone propionate the authors state, "In agreement with previous workers the administration of low doses of testosterone propionate 0.1 mg. daily for 20 days caused inhibition of testicular growth." It is now obvious that this dosage contrary to being "low" was markedly excessive.

The results obtained in the 2 groups treated with 20γ doses are noteworthy since they offer an opportunity to compare the reactions of intraperitoneal and subcutaneous administrations. Working with females, Israel, Meranze and Johnston (18) observed a counteracting effect of the liver to estrogens and concluded that subcutaneous administration would, therefore, be more potent. From tables 2 and 3 it appears that the seminal vesicles and testes of our 2 groups were roughly in the same weight class after treatment. This would seem to indicate that for testosterone propionate when used in this particular dosage in the male, the effect of the liver is negligible.

The probable mode of action of testosterone propionate on the gonad requires consideration since until now only its depressing effect was considered to be carried out through the hypophysis (19–23). On the basis of more recent work it now appears that testosterone propionate can also evoke stimulating effects through the pituitary (5, 24) which in the immature female leads to premature ovulation (25, 26). Whether this stimulatory hypophyseal effect can occur in the male is as yet undetermined.

At this time, it seems safe to say that testosterone propionate affects all accessory sex organs directly. Its effect upon the testis, however, is both direct and indirect. In small doses as given in these experiments, i.e., 5γ for 10 days between 22 and 32 days of age, testosterone propionate may mildly stimulate the testis and lead to proliferation of the germinal epithelium. In contrast to the gonadotropic hormones the interstitial tissue is not increased. This speaks in favor of the elaboration of the androgens by the interstitial tissue. This direct stimulating effect upon the testis is confirmed by studies which disclosed that androgens given immediately after hypophysectomy could maintain spermatogenesis in scrotal testes (27–30) and by the fact that immature hypophysectomized rats receiving 2 mg. testosterone propionate and dehydroandrosterone acetate proceeded towards complete spermatogenesis (31). At this time, however, a conservative attitude regarding the testicular growth stimulating effect of small doses of testosterone propionate is recommended since it has been observed (32) that this response is not fixed in a definitely constant manner. For example, in one group studied 10γ daily led to an increase in testicular size. In the series herein reported 10γ neither increased nor depressed the testis. In still another

series 5γ failed to stimulate testicular growth but 2.5γ did result in a 12% increase. Because of this inconstancy which results greatly from biological variation, it appears safer to reserve any conclusion regarding the testicular growth stimulating function of testosterone propionate for the future. It is important at this time to know that one can use androgenically potent doses of testosterone propionate without inducing testicular damage. When doses are increased in the intact animal, the pituitary gland is depressed so that the testis becomes inhibited in its growth. If dosage is still further increased the testis undergoes atrophy and takes on a picture of degeneration.

Finally, the non-deleterious and perhaps stimulating effects of small doses of testosterone propionate upon the testis is in keeping with the common knowledge that adolescent and normal adult males carry within their blood definite concentrations of androgenic substances. Yet, the presence of these blood androgens neither prevents testicular growth at puberty nor does it cause testicular atrophy in the healthy adult.

SUMMARY

Small doses of testosterone propionate (5γ) injected into male albino rats daily from 22 to 32 days of age led to a probably significant increase (by 17%) in the weight of the testes. Proliferation of the germinal epithelium was stimulated but maturation of spermatozoa remained unaffected. With 10γ similarly administered testicular weight remained unchanged. With higher doses at this age testicular weight was progressively decreased reaching a minimum (−51%) with 50γ. All doses progressively higher than 50γ continued to depress testicular weight but the degree of depression was in inverse proportion to the size of the dosage. Microscopically, all testes so treated showed signs of increased germinal proliferation. No evidence of premature maturation was observed. A daily dose of 2.5 mg. resulted in degeneration and sloughing of the germinal epithelium.

Doses (e.g., 50γ) found to be depressing when used for short periods in young animals failed to depress the testis when continued over longer intervals. This was considered to be due to the added body growth which diluted the hormonal concentration sufficiently to prevent its deleterious effects.

The following modus operandi is considered as a working hypothesis. Testosterone propionate in small dosage stimulates proliferation of the germinal epithelium. Upon increasing the dose testosterone propionate depresses the pituitary gland which in turn inhibits testicular growth and spermatogenesis so that with markedly excessive doses, degeneration follows. As the dosage is still further increased the gigantic proportions of the reacting secondary sex organs become so striking that much of the injected hormone is utilized merely for their maintenance. This diminishes blood androgen concentration thereby depressing to a lesser degree the anterior lobe of the pituitary. The testis is thus correspondingly spared.

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EFFECT OF ORAL ADMINISTRATION OF METHYLTESTOSTERONE ON FATIGUE IN EUNUCHOIDS AND CASTRATES

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One of the most constant effects of testosterone treatment in patients with diminished or abolished testicular hormone production (eunuchoids, castrates) is the feeling of increased working capacity and endurance. A few attempts have been made to establish a physiological explanation for this. Hoskins (1) compared the voluntary activity of 16 castrated male rats with 16 normal rats. The animals were placed in a revolving cage, and the number of revolutions recorded. It is estimated that about 95% of the total activity of the animal is registered by this method. He found that castration diminished the voluntary activity in male rats about 60%. Gans and Hoskins (2) measured the absolute strength and the performance of the gastrocnemius muscle in 19 male rats, 95 to 277 days after castration. The sciatic nerve was stimulated by induction shocks until complete fatigue occurred. The average performance of the control group was 107.924 gm. cm., that of the experimental group only 81.277 gm. cm., i.e. 25% less. The absolute strength per gm. of muscle was approximately the same in each group, although the weight of the gastrocnemius muscle was greater in the castrated than in the control animals. It may be mentioned that Miley (3) was able to produce similar results with regard to voluntary activity performance, fatigue, and muscle weight in female rats by ovarian extirpation. The absolute strength per gm. muscle was diminished about 40% by oöphorectomy.

In contrast to the increased muscle weight following castration obtained by these authors, Papanicolaou (4) found a generalized muscular enlargement, especially in the temporal muscles, after prolonged administration of androgenic hormone (testosterone propionate) in castrated immature male and normal adult female guinea pigs. In adult female guinea pigs a muscle hypertrophy could also be produced by application of gonadotropic hormone (follutein).

In experiments such as performed by Gans and Hoskins (2), where fatigue was produced by electrical stimulation of the sciatic nerve, the fatigue is located in the motor end-plates. Such fatigue cannot be reproduced in man by voluntary action. Mosso has shown that electrical stimulation, applied immediately after complete fatigue of voluntary finger movements in the finger-ergograph, produced contractions. Thus fatigue of man in this type of work must be localized in the central nervous system.

Reid (5) concludes that the fatigue of the muscles during voluntary effort is due to an inhibition in the central nervous system brought about by afferent nerve impulses arising in the muscles. This is especially true for static work. There is evidence that fatigue in voluntary action develops first in the sensory centers. In any type of

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fatigue in man central and peripheral functions are involved, in some types of work the central nervous processes prevail, in other types the peripheral functions. Fatigue may be due to different physiological functions even in the same type of work, when performed with big or small loads, or at high or low speed. (For bibliography see Simonson, 6.)

It seemed important to investigate whether or not the endurance in man for muscular work could be increased by testosterone, and, if so, in what types of work. The following tests were applied: *a*) absolute muscle force; *b*) endurance in a heavy type of dynamic work, leading to fatigue in not more than 3 minutes; *c*) static work with the same load as used in *b; d*) finger-erograph; *e*) fusion frequency of flicker.

The endurance in type *b* is much more dependent on circulation and respiration than it is in the types *c* and *d*, where fatigue is primarily due to the inhibition produced by sensory impulses.

The fusion-frequency of flicker is that rate of successive light stimuli necessary to produce an effect equal to continuous illumination. The fusion frequency indicates the maximum number of impulses the retino-cortical system is able to receive per unit of time. Thus the fusion frequency may be looked upon as an expression of the excitability of the retino-cortical system. The following fundamental functions are involved in the fusion frequency: chronaxia, latent period and refractory period of the retino-cortical system. As the fusion frequency is one of the most important sensory functions, the results may be generalized to the whole central nervous system as shown by other experiments. We have found a constant and consistent decrease of fusion frequency in physiological as well as in pathological fatigue (7, 8). Thus the fusion frequency of flicker indicates the degree of fatigue and the resistance against fatigue of the central nervous system.

The pulse rate and lung ventilation during and after dynamic and static work, and the recovery speed of working capacity were determined with each test.

METHOD

a) *Finger-ergograph.* The finger-ergograph was similar to Mosso's ergograph. The forefinger was loaded with 2 kg. and moved until complete fatigue at a rhythm of 60 lifts per minute. The amount of work was calculated from the ergogram in m-kg.

b) *Dynamic work.* Dumb bells of 8 or 12 pounds were lifted one meter, with straight arms, at a rate of 20 per minute. The work was continued until complete fatigue. Although this type of fatigue is subjective, as it is in any voluntary action, there are signs of the developing fatigue. At the end of the exercise, 20 to 30 seconds before fatigue, auxiliary muscle groups are stimulated, and the type of movement changes. For the test that load was chosen which produced fatigue within 2 to 3 minutes. Thus the subject *Mo* was examined with 12 pounds, and the subjects *Ne* and *Y* with 8 pounds.

c) *Static work.* The subject had to hold the same load as in the dynamic work test with the straight right arm in horizontal position until complete fatigue. Here, too, fatigue signs may be observed shortly before fatigue is complete; tremor and stimulation of auxiliary muscles. It is important that the subject does not change his posture during the experiment.

d) *Recovery of working capacity.* In the types *b* and *c* and in the subject *Y* in type *a*, after complete fatigue a recovery interval of 2 minutes was allowed. The subject was then asked to repeat the test. The second performance is lower than the first, because the interval of 2 minutes is too short for complete restitution, but this difference depends on the recovery speed. At low recovery speed the second performance would be much lower than the first, at high recovery speed the difference between

the performances would be small. In case the second performance equals the first (= 100) the interval of 2 minutes was sufficient for complete restitution. The recovery speed is expressed by the relative value of the second performance in percentage of the first.

e) Heart rate. The heart rate was measured each 15 seconds for 10 seconds during the work and recovery through a stethoscope fixed to the chest at the apex area.

f) Pulmonary ventilation. This was measured by means of a dry gasometer. A rubber valve separated the inspired from the expired air. Before exercise the resting value was measured after a sufficient resting time. During the work the ventilation was measured every 30 seconds, during the recovery, every minute. The measurement of the recovery of the second performance was measured until the resting value was reached. The subject was in a sitting position during the recovery period. The values were reduced to normal atmospheric conditions.

g) The absolute muscle strength. This was measured by means of a spring coil dynamometer. The subject standing in a bowed position, seized the handle with both hands and pulled upward. This test was repeated from 3 to 4 times, and the maximum value was taken. In this type of pull a large number of muscle groups are involved (arm, shoulder, trunk, leg), so that the value may be regarded as an index for general muscle power.

h) Fusion frequency. A known rotator arrangmeent was used, by means of which a beam of light from an electric bulb (25-watt Mazda) was interrupted by a rotating disk with 4 identical openings. The subject was seated with his head placed on a chin rest so that the distance from the illuminated area to the eye was kept constantly at one meter. The subjects were examined under the following standard conditions: the vertical intensity of illumination of the surrounding field at the plane of the illuminated area was 2 foot candles; the size of the illuminated area was 100 sq. mm., equivalent to a visual angle of ½°. Two rotating disks were used, opening A with a relative duration of the light flashes of 64% of the total cycle (flash interval plus dark interval), and opening 4 with a relative duration of the light flashes of 17.7% of the total cycle. The intensity of illumination of the test patch was 0.009 candle power per square centimeter for opening 1, and 0.0039 candle power for opening B. Binocular central vision was used. The accuracy with which the values can be reproduced is high; the variation of the values, taken in the same experiment, does not exceed, as a rule, 1 to 1½ cycles. The daily variations do not exceed, as a rule, 3 cycles, provided that the subject's condition is unchanged.

SUBJECTS

1, *Ne.* Weight, 164 lb.; height, 70 in.; arm span, 73 in.; age 26 yr.; vision, normal; vital capacity, 4.5 l.; occupation, clerk. A typical eunuchoid with feminine distribution of fat, feminine hair pattern over pubis, with no coarse hair on face. Testicles undescended. No prostatic development.

2, *Mo.* Weight, 172 lb.; age, 47 yr.; vision, normal; vital capacity, 3 l.; occupation, male nurse. Both testicles removed for tuberculosis at 35 years.

3, *Y.* Weight, 125 lb.; height 62 in.; arm span, 54 in.; age, 21 yr.; vital capacity 2.4 l.; vision corrected to normal; student. Typical eunuchoid with both testicles descended but markedly hypoplastic. Marked underdevelopment of external genitalia. Feminine hair growth and fat distribution.

4, *Bu.* Weight, 145 lb.; height, 68 in.; age, 36 yr.; vision normal; lost one leg by accident, therefore, tests *b*) and *g*) could not be performed. Bilateral castration for tuberculosis at the age of 32 years.

TREATMENT

All 4 patients were denied treatment of any kind for a period of one month in

preparation for the tests. At the end of this time each patient was examined with all of the tests, on 2 occasions with an interim of approximately 3 days. Treatment was then instituted. Twenty-five milligrams of methyltestosterone in tablet form were administered orally 4 times daily for a period of 3 weeks. At the end of this period the tests were repeated twice at two sessions. Thus any influence of training on the results obtained, may be excluded.

In the administration of testosterone in hypogonadism 4 routes of administration have been found effective: (*a*) testosterone propionate in oil solution used in subcutaneous injections; (*b*) free testosterone in ointment massaged into the skin; (*c*) implants of testosterone pellets (9); (*d*) ingestion of methyltestosterone by mouth in tablet form.

The choice of method depends upon: the physiological response of individual patients to the route of administration; the economic status and vocation of the patient; the attitude of the patient and his cooperation with the form of treatment.

Methyltestosterone administered by mouth gives great promise of becoming a most acceptable method of treatment. All of the results obtained by the other methods may be duplicated with the oral administration. Six to eight times the injection or ointment dose is required. Provided the expense to the patient is not too great the oral route of administration will no doubt receive widespread use.

RESULTS

Finger-ergograph. The results are shown in table 1. In subject *Mo* the amount of work performed until fatigue was the same before and after treatment; but in the 3 other subjects the performance was increased from 20% to 122%. The average in-

TABLE 1. INFLUENCE OF METHYLTESTOSTERONE TREATMENT ON ENDURANCE AND PERFORMANCE (AMOUNT OF WORK)

Subject	Type of work	Endurance (sec.)		Performance M·kg.		Increase in percentage of values before treatment	
		before	*after*	*before*	*after*		
Ne	Finger-ergograph	50	48	2.00	2.40	+20.0%	
Mo		180	90	3.60	3.54	− 1.0%	
Y		52	70	1.04	2.32	+122.0%	Average
Bu		60	110	3.71	5.21	+40.5%	+45.4%
Ne	Dynamic work 8 lb.	115	180	276	432	+56.5%	
Mo	Dynamic work 12 lb.	200	415	720	1436	+107.0%	Average
Y	Dynamic work 8 lb.	90	128	216	308	+43.1%	+68.5%
Ne	Static work 8 lb.	45	52	—	—	+11.6%	
Mo	Static work 12 lb.	20	30	—	—	+50.0%	
Y	Static work 8 lb.	12	24	—	—	+100.0%	Average
Bu	Static work 8 lb.	75	77	—	—	+2.8%	+41.1%

crease was 45.4%. In subject *Bu* the increased performance was due to increase of endurance only. In the subjects *Ne* and *Y*, the increase of performance was due to an increase of the contraction height, and in the subject *Mo*, the increase of contraction height compensates for a decrease in endurance.

Dynamic work. Table 1 shows that the duration and the amount of dynamic work performed until fatigue, increases from 42.1% to 107% (average about 68.5%). The treatment improved especially the stronger subjects. The absolute and relative increase is more pronounced the longer the absolute period before the treatment.

Static work. The endurance in static work is not increased in subject *Bu* (+2.8%); slightly increased in subject *Ne* (+11.6%); and largely increased in subjects *Mo* (+50%) and *Y* (+100%). The average increase of endurance in static work is about 41.1%. It is interesting that the increase of endurance in static work is in inverse ration to the absolute performance. This is true in regard to the absolute duration in seconds as well as to the relative increase in percentage of the performance before treatment. Thus the testosterone treatment benefits weak subjects most importantly with respect to static work.

Absolute muscle strength. The absolute muscle strength was not altered in subject *Ne* (120 kg. before and after treatment); it increased in subject *Mo* from 117.5 to 125.0 kg. (+7.5 kg. or +6.5%); in subject *Y* from 75.0 to 87.5 (+12.5 kg. or +16.5%). The increase of absolute muscle strength is most pronounced in the weakest subject, *Y*, like the increase in static performance. Thus the increase of static performance seems to be partially due to the increase of absolute muscle strength. The increase of absolute muscle strength is comparatively slight. See Gans' and Hoskin's results, who found no decrease by castration.

Recovery speed. Table 2 shows a definite improvement of recovery speed. Most uniform is the increase of recovery speed in static work. In the 2-minute interval after

TABLE 2. INFLUENCE OF TESTOSTERONE TREATMENT ON THE RECOVERY SPEED OF WORKING CAPACITY

Subject	Type of work	First performance		Second performance after 2-minute interval		Recovery accomplished in % of first performance	
		before	*after*	*before*	*after*	*before*	*after*
Bu	Finger-ergograph	3.71	5.21	0.88	1.98	23.7	38.0
Y		1.04	2.32	0.53	1.98	50.8	85.2
Ne		—	2.40	—	1.60	—	66.6
Y	Dynamic work 8 lb.	216	308	125	168.4	58.0	54.7
Ne	Static work 8 lb.	45″	52″	32″	44″	71.2	84.5
Mo	Static work 12 lb.	20″	30″	15″	30″	75.0	100.0
Bu	Static work 8 lb.	75″	77″	53″	72″	70.6	93.5
Y	Static work 8 lb.	12″	24″	8″	24″	75.0	100.0

the first performance, the recovery is accomplished from 70.6 to 75% (average 72.7%) before the treatment, and from 84.5 to 100% (average 94.5%) after the treatment. There is also an improvement in the recovery speed in the finger-ergograph work. On the other hand the recovery speed remained the same in dynamic work for subject *Y*. In this subject the increase of performance in dynamic work was least pronounced. In other subjects the recovery after dynamic work has not been measured; an increase seems to be possible. We intend to investigate the recovery speed in dynamic work more closely. It is possible that the improvement of recovery speed in static work and finger-ergograph work is to a certain degree due to acceleration of recovery processes in the central nervous system.

Pulse rate. Table 3 shows the maximum increase of pulse rate above the resting value (excess pulse rate), in dynamic work, static work and finger-ergograph. The improvement in dynamic performance by training coincides with an increase of the stroke volume of the heart and a decrease of the heart rate. Therefore a decrease of the pulse rate in dynamic work must be considered a favorable symptom. Table 3 shows that the maximum increase of pulse rate during dynamic work is diminished about 12 beats per minute after testosterone treatment. It is probable that the improvement of endurance in dynamic work is partially bound to the increased effi-

TABLE 3. EXCESS PULSE RATE AND LUNG VENTILATION BEFORE AND AFTER TESTOSTERONE THERAPY

Subject	Type of work	Pulse rate, (beats/min.) maximum increase above resting rate		Excess ventilation Work-minute	
		before	*after*	*before*	*after*
Ne	Dynamic 8 lb.	54	42	22.8	22.5
Mo	Dynamic 12 lb.	53	42	31.5	29.9
Y	Dynamic 8 lb.	66	54	17.7	19.3
Ne	Static 8 lb.	30	36	19.8	24.0
Mo	Static 12 lb.	35	42	41.0	28.1
Y	Static 8 lb.	—	—	28.8	18.8
Bu	Static 8 lb.	30	21	19.2	16.6
Ne	Finger-ergograph	24	18	—	—
Mo		12	12	—	—
Y		24	24	—	—
Bu		15	9	—	—

ciency of the circulation. The circulatory factors are not so essential in static work and in work with the finger-ergograph as they are in dynamic work. Compared to dynamic work, the increase of blood flow is only slight. The increase of pulse rate is more due to the radiation of nervous impulses than to chemical regulation of blood flow. The nervous regulation of the pulse is not so closely adapted to the performance as is the chemical regulation. The results of testosterone treatment on the excess pulse rate in static work and finger-ergograph are not uniform.

Pulmonary ventilation. The excess ventilation (total ventilation minus resting ventilation) was calculated for the total duration of work plus recovery period. This value was divided by the duration of work in minutes. Thus the excess ventilation necessary for the duration of one minute of work was obtained. Table 3 shows these

TABLE 4. FUSION FREQUENCY OF FLICKER

Subject	Before treatment		After treatment	
	Opening B	Opening A	Opening B	Opening A
Ne	46.0	83.2	49.0	89.4
Mo	36.8	73.4	38.8	76.0
Bu	43.0	91.6	50.0	95.2
Y	46.0	82.0	48.4	94.0

values for dynamic and for static work. The ventilation has not been measured in the finger-ergograph work. In dynamic work, the excess pulmonary ventilation is not influenced by testosterone treatment. In static work there is a tendency to decrease by testosterone treatment (decrease in 3 subjects, increase in one subject, mean value 27.2 before, 21.9 l. after treatment).

The fusion frequency of flicker. The fusion frequency of flicker is increased in both opening B (relative duration of flashes 64%) and opening A (relative duration of flashes, 17.7%).

DISCUSSION

Our results show a definite influence of testosterone therapy on the endurance and performance in different types of work. Thus these results may be regarded as a physiological background for the observations that patients feel more capable for work after testosterone treatment. The effect differed with the subjects and the types of work. A great many physiological functions are involved in muscular work and

endurance, only a limited number of which could be investigated simultaneously. But obviously they are differently influenced in our subjects. This is not difficult to understand; although the diminished production of sexual hormone is a common feature of our 4 subjects, there are also considerable constitutional differences. It may be mentioned that Gans and Hoskins (2) obtained large individual differences in the effect of castration of rats in regard to activity and muscular performances although conditions may be much better standardized in animal experiments than in experiments on man. We regard the increase of fusion frequency as one of the most interesting results. It indicates an increased resistance of the central nervous system against fatigue; it accounts to a large degree for the increased endurance in static work and in finger-ergograph, and it is, as far as we know, the first observation of improvement of an important central nervous function by testosterone treatment. In connection with this it may be mentioned that Katai (10) found measurable atrophic changes in the brain and spinal cord of white rats deprived of their testes. These results suggest control of testosterone therapy by means of combined exercise tests.

SUMMARY

In 4 male subjects with diminished production of sexual hormone (2 eunuchoids, 2 castrates) the following effects of 4 weeks testosterone treatment were obtained: increase of muscular performance, *a*) in a type of dynamic work in the average of +68.5%; *b*) in a type of static work in the average of +41.1%; *c*) in work with the finger-ergograph in the average of +45.4%. The absolute muscular strength was slightly increased. The recovery speed increased in static work and in finger-ergograph work. The maximum increase of pulse rate during dynamic work was diminished indicating more efficient circulatory system. The pulmonary ventilation was slightly decreased in static work and unchanged in dynamic work. The fusion frequency of flicker was increased thus pointing to an increased resistance of the central nervous system against fatigue.

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THE TREATMENT OF VITILIGO BY LOCAL INJECTIONS OF MELANOPHORE HORMONE

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WE PREVIOUSLY REPORTED success in the treatment of vitiligo with intradermal injections of the melanophore hormone. Two additional cases of vitiligo successfully treated in the same way are reported here.

Case 1. O. C., a girl, aged 12, complained of vitiligo of 6 months' duration. This appeared coincidentally with the secondary sex characters, showing first as a small patch just above the manubrium. Shortly after this the hair began to become gray at the right parietal area.

At the time of the institution of treatment the patch of vitiligo covered a wide area limited by the lower border of the lower jaw superiorly, the posterior border of the sternomastoid muscle laterally, and on a line from the chin to the manubrium anteriorly. Its contour was very irregular, with many extensions all around, one of which reached as far as the back of the neck, where several small areas were independent of the main patch. The rest of the body appeared free from vitiligo.

It is interesting to note, although the fact has nothing to do with vitiligo, that this patient presented a hypoplastic constitutional type. She began to menstruate at 12 years and 2 months of age while still under our treatment, the duration of which was 3 years including the periods of rest. Menses were very irregular and prolonged amenorrheic periods are reported to have occurred.

The patient was of short stature until puberty, when her height markedly increased along with the appearance of vitiligo and the graying of the hair. Otherwise, the personal history is unimportant.

The mother of the patient was of hypoplastic type; her menses appeared at 15 years of age, are normal at present except for dysmenorrhea.

Treatment. Two fresh hypophyses a day, orally, were prescribed. This treatment lasted 14 months. At the end of this time the patch of vitiligo was reduced to about one-half its former size; there remained, however, a small patch 2 cm. in diameter at the back of the neck. Oral treatment was then stopped as a consequence of the difficulties in obtaining fresh hypophyses. Instead, the intradermal injection of the melanophore hormone was resorted to (200 U once or twice a week for 20 months). Ninety-four injections were given in all.

The main patch of vitiligo had become about one-half its original size at the end of oral treatment, and a small patch still persisted at the back of the neck. The area of gray hair over the parietal area had remained unaltered. Shortly after beginning the intradermal injections—given locally into the persisting area of the main patch—a change of color was noted, e.g., the skin, which had formerly been of a dull white tinge, became of a pinky hue that greatly reduced the contrast with the adjacent skin. Then slowly, a normal pigmentation began at the periphery and proceeded toward the center of the area, while within this there appeared some pigmented, rounded spots that extended out slowly and eccentrically. Meanwhile, the small zone of vitiligo at the back of the neck entirely disappeared and the right parietal area of grey hairs was markedly reduced, in spite of receiving no local treatment at all. At present, the vitiligo consists only in a small patch of very irregular limits and less than 2 sq. cm. in its area.

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Case 2. R. G. de U. was a married woman aged 24, whose chief complaint was vitiligo spread over widely different parts of the body. The course had been progressive for the past 7 years. Its beginning was connected by the patient with a serious emotional shock.

The patient had had only one pregnancy, which was badly tolerated, with severe vomiting during the 9 months, headaches, somnolence, sensitiveness to cold, and a marked reduction in weight. The already present vitiligo spread rapidly and involved new regions of the body. Following delivery, the patchy discoloration of the skin continued until the patient came under our observation. The last patches to appear dated back to a year previously and affected the face (chin, left periorbital region and later the right one). There were also various localizations on the body other than those about the face: thighs, knees, elbows, abdomen,

Fig. 1. *Case 1* BEFORE TREATMENT. Fig. 2. AFTER ORAL TREATMENT WITH FRESH HYPOPHYSIS. Fig. 3. AFTER LOCAL TREATMENT WITH THE MELANOPHORE HORMONE. Fig. 4. *Case 2* BEFORE TREATMENT. Fig. 5. AFTER TREATMENT.

thorax, hands and fingers. The feet were the only regions spared. Except for the vitiligo, the physical examination showed nothing abnormal.

The infantile development of the patient was good. The first teeth were delayed in appearance. First menses appeared at 12 years; the flow was abundant and the intervals regular. She had diphtheria at 5 years, recurring intestinal infections with fever from 7 to 10 years of age. A paternal aunt has had vitiligo for 10 years; she is now 50.

Treatment. Intradermal injections of the melanophore hormone were given in dosages of 200 U each. Because of the patient's irregularity in coming to the clinic this treatment had to be carried out weekly and biweekly. All the injections—a total of 72 in a year—were given into the same patch of vitiligo about the left elbow.

Shortly after the treatment was started the dull white hue of all of the patches of vitiligo diminished and turned to a light pink. Later, the white areas about the face began to turn to normal, first at the chin, then at the left periorbital region, and finally at the level of the right periorbital region. The relief increased until at present all the patches about the face are entirely restored to normal, while the patches over the rest of the body have very much the

same size as before, even at the level of the treated area (left elbow), showing only the above-mentioned changes in their hue, which reduces the contrast with the neighboring normal skin. The condition, which had an extensive tendency before, has now stopped in its progression.

COMMENT

In the previous paper on this same subject we reviewed several hypotheses in an attempt to explain the mechanism through which such results could be obtained. These might be the outcome of (*a*) spontaneous relief; (*b*) a reflex irritative process influencing somehow or other the cutaneous pigmentation; (*c*) a specific action of the melanophore hormone.

We think the first hypothesis can be discarded from a practical point of view since treatment proved to be effective more than once. The second hypothesis is disproved by the fact that some improvement was also observed to take place about areas far distant from the treated area. This fact also supports the view that the hormone exerts a general effect.

Finally, the beneficial effects of the oral administration of fresh hypophyses, as seen in the first case, have led us to think it possible that the digestive processes do not destroy all of the melanophore hormone in man, a fact which we have experimentally confirmed in frogs.

NOTES AND COMMENTS

THE PRESENCE OF A GONADOTROPIC SUBSTANCE IN THE URINE OF NON-PREGNANT FEMALE MACAQUES

A SUBSTANCE causing a positive Aschheim-Zondek test in rats or mice and a positive Friedman test in rabbits has been shown to be present in the urine of the pregnant chimpanzee[1,2,3] and the pregnant macaque.[4] However, no observations have been reported upon the presence of a gonadotropic principle in the urine of non-pregnant monkeys or apes. It is well known, however, that the non-pregnant human female excretes in the urine at different times during the menstrual cycle varying amounts of a gonadotropic substance which will cause ovulation in the rabbit. Therefore it was thought probable that non-pregnant monkey urine should also contain a similar active substance.

Methods. The animals used in the experiment were two adult macaques of uncertain age. One was used over a period of two years, 1938–39 and 1939–40. The second was used only during 1939–40. The first animal had an infant, born in 1937 in the animal house, but had ceased suckling the baby at the time the first experiment was begun.

The animals were caged separately. The galvanized iron cage bottoms and the screenings above this were painted with aluminum paint. They were fed and watered once a day over a period of 2 to 3 hours and then all food and water was removed. The urine was caught in a 500 cc. flask, placed in a large thermos bottle filled with ice. No other preservative was used.

The method of precipitation and extraction of each 24-hour urine specimen was essentially that of Levin and Tyndale[5] with a few modifications. The urine was centrifuged to remove debris and brought to approximately PH 5 with acetic acid. Twenty cc. of 10% aqueous tannic acid were added per liter of urine and well mixed. The urine was now allowed to stand in the cold for 2 hours. The precipitate was collected by centrifuging, washed twice with acetone, transferred to a 50 cc. centrifuge tube and dried, using a current of air drawn over $CaCl_2$. When a 4-day collection was desired the first 3 daily samples were stored in the cold, dry, and well stoppered. The dried material was pulverized before extraction with a weakly alkaline solution. NaOH was used to keep the reaction approximately at PH 8 using alkacid paper for the indicator.

The total volume of the extracting fluid was 8 to 11 cc., and the time of extraction, carried out at 0° C. in a beaker of ice and water, approximately 30 minutes. The residue was removed by centrifuging, leaving a deep brown supernatant fluid, fairly clear. A drop or two of N/20 acetic acid usually was added to get a PH of about 7 and the extract was injected into the ear vein of a rabbit. Occasionally such an extract was crude enough to kill the animal but in general a healthy rabbit showed no ill effects. A positive result was represented by demonstrating fresh ovulation points in the ovary 18 to 20 hours after injection.

Results. The urine collected represented approximately 20-hour quantities, minus about one-third due to loss from evaporation in the cage bottom on the way to the collecting funnel. The results are seen in the chart below. Only positive tests are shown. Many negative experiments and unsuccessful ones (due to some error in collecting extracting, death of rabbit, etc., etc.) are omitted since this report is merely a preliminary note of interesting observations made somewhat as a sideline during the course of other work. It is, therefore, to be emphasized

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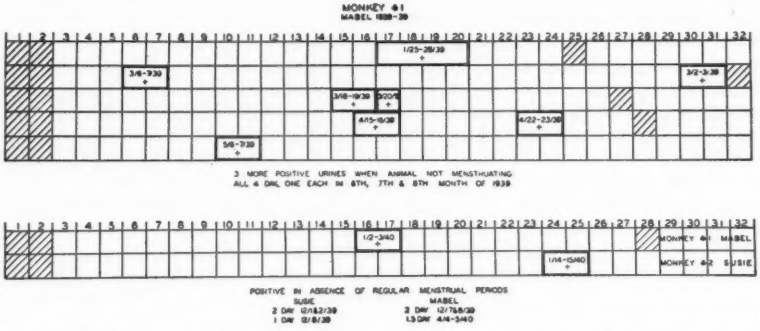
that for the same reasons periods where no positive results are shown on the chart by no means are to be considered as representing times when the urine did not contain the active principle.

Discussion. It is interesting to note that over a period of months positive experiments with 2 days of urine were obtained in *monkey 1* at all stages of the cycle. The only positive results obtained using only 1-day collections were recorded about mid-cycle, one 17 days after a spontaneous menstruation and one 14 days after bleeding which followed an injection of 100 mg. of progesterone given over a period of 3 days. Positive 2-day experimental results were found occasionally during amenorrhea. Two-day negative experimental results were found at



all times during the cycle but have much less weight than positive results, because of the circumstances of the assay.

During the summer when ovulation in the macaque does not occur, as both Corner and Hartman have shown, positive results probably required more urine than in the winter. This observation, however, is only a probability, because the assay object, the rabbit, is not as desirable a tool in the summer as during the cooler months. Four-day positives were however found in mid-summer when ovulation ceases and amenorrhea is common. No 2-day positives were found during the summer, but on the other hand very few were attempted.

Summary. By properly collecting and treating the urine obtained from two female non-pregnant macaques it was possible to demonstrate the presence in that urine of a gonadotropic substance which will cause ovulation in the rabbit.

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INFLUENCE OF THYROIDECTOMY ON FAT DEPOSITION IN THE RAT

THERE IS NO DOUBT but that hyperthyroidism, produced by overactivity of the gland or the administration of active thyroid material to man or animals, increases the energy expenditure more than the appetite and leads to a reduction in deposit fat. That hypofunction of the thyroid gland may cause the opposite effect and contribute to the causes of obesity is quite another matter. Although it is well known that patients with myxedema are not obese as a rule the idea persists in the clinical literature that hypothyroidism may be a cause of obesity. In connection with observations made for other purposes an opportunity was afforded of examining this question in a controlled experiment with animals.

When they were 170 days old 3 groups of male albino rats were placed upon a relatively high fat diet.[1] One group was thyroidectomized and another had 0.1% U.S.P. dessiccated thyroid added to their food. All of them were killed 310 days later and the body fat (total lipids) determined. On the diet which was used adult rats tend to acquire quantities of de-

TABLE 1. INFLUENCE OF THYROIDECTOMY UPON BODY FAT

Thyroidectomized			Controls			Thyroid fed		
Body weight			Body weight			Body weight		
Initial[1] gm.	Final[2] gm.	Fat %	Initial gm.	Final gm.	Fat %	Initial gm.	Final gm.	Fat %
279	468	6.0	288	490	32.4	285	310	14.7
265	425	6.4	267	450	29.5	265	305	14.3
260	468	6.0	264	540	35.9	262	301	13.8
253	392	7.4	263	420	30.4	250	280	13.4
253	410	6.3	252	390	23.6	250	286	15.1
249	470	6.5	251	455	35.2			
Av. 260	438	6.4	264	457	31.1	262	296	14.2

[1] 170 days old.
[2] 480 days old, the body fat determinations were made at this time.

posit fat much higher than on the stock diet. The body fat had reached a level (table 1) of 31.1% in the control group when the experiment was terminated.

The thyroidectomized group weighed almost as much as the controls and appeared to be as plump but had a body fat content of only 6.4%, just a fifth of that found for the controls. Their plump condition was undoubtedly due to myxedematous changes in the tissues.

The inclusion of active thyroid substance in the diet produced the usual decrease in body fat known to follow hyperthyroid activity. The fat content was less than half that of the controls.

Summary. Thyroidectomy in the adult male albino rat results in a marked reduction in the amount of body fat in comparison with unoperated controls of the same age when they are killed 10 months later. In spite of the low fat content the thyroidectomized rats do not appear less plump nor do they weigh appreciably less than the controls.

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OCCURRENCE OF ESTROGENS IN THE OVARIES OF THE WINTER FLOUNDER*

ALTHOUGH their physiological significance is far from clear, the presence of estrogenic substances in the ovaries of marine and fresh water vertebrates and invertebrates is now fairly well established.[1-6] The concentration of estrogens appears to be low in these forms but the use of crude extracts, from necessity because of limited amounts of ovarian tissue available, has precluded the possibility of carrying out precise, quantitative, assays.

Materials and methods. The present study concerns the preparation and testing of extracts of the ovaries of the winter flounder (*Pseudopleuronectes americanus*) for their estrogenic properties. The ovaries were obtained through the courtesy of Dr. Paul Galtsoff of the U. S. Bureau of Fisheries. Forty large ovaries were taken from flounders netted off the Woods Hole Station in February 1940. The eggs appeared to be mature in most cases. The whole ovaries were packed into pint jars containing 95% ethyl alcohol and shipped to Charleston where extraction was begun immediately. The ovaries were ground thoroughly in a mortar and the original alcohol filtered off and saved. The ground tissues were dried in an oven set at 37° C. and then re-extracted with 4 times their volume of alcohol. The alcoholic filtrates were combined and concentrated by distillation under reduced pressure. The tissues were then re-extracted with acetone and ether respectively, again using 4 times the tissue volume and making two extractions with each extractive. All extractions made lasted for 48 hours each. The alcoholic concentrate appeared as a virtually black, tarry substance which was extracted once with acetone and discarded. The acetone concentrate appeared as an orange colored oil which became semi-solid upon standing. The ether concentrate appeared as a dark brown oil which set into a solid fat on standing. It was supposed that after thorough extraction with alcohol and acetone little or no more fatty material would be extractible with ether. However, the yield with ether was considerable and, as the results indicate, a considerable amount of estrogenic material was contained in the ether concentrates. The acetone and ether concentrates were tested separately. The yields of crude extract were: 22 cc. for the combined acetone concentrates and 62 cc. for the combined ether concentrates. The tissue residue following all extractions weighed 895 gm. in the dry state. The acetone fraction was tested without dilution. The ether fraction was tested after being diluted with just enough corn oil to make it flow.

Mature, ovariectomized rats were used as test animals. Ten days were allowed to elapse following ovariectomy before extracts were tested. Vaginal smears were taken once daily during the injection period, great care being exercised not to traumatize the vaginal epithelium. The possibility of obtaining false positive smears by rough smearing procedures makes it most important that this part of the procedure be adequately controlled. The procedure and results are summarized in table 1. It will be noted that most animals received extract applications direct to the vagina. Since these extracts contain, at best, but small amounts of estrogen per unit of extract, the vaginal application procedure proved many more times as sensitive as the subcutaneous method of injection. For controls 10 ovariectomized animals of the same age were given vaginal applications of corn oil for a like number of days. In all instances the control group showed no disturbance of the diestrous phase established

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TABLE 1. EFFECT OF ESTROGENS FROM FLOUNDER OVARIES ON THE RAT VAGINA

Rat	Age	Daily dosage	Fraction used	Route	Day of 1st estrous smear	Vagina[1]	Injection period
	days	*cc.*					*days*
A1	110	0.50	ether	subcut.	0	0	10
A2	113	0.50	acetone	subcut.	0	0	10
A3	113	0.50	acetone	subcut.	0	0	10
A4	113	0.50	acetone	vagina	5	12	10
A5	113	0.50	acetone	vagina	5	10	10
A6	93	0.50	acetone	vagina	4	6	6
A7	93	0.50	acetone	vagina	4	8	6
A8	93	0.50	ether	vagina	4	4	6
A9	93	0.50	ether	vagina	4	5	6
A10	113	0.50	mixed	vagina	4	8	6

[1] Figures indicate number of newly grown cell layers in vaginal epithelium. For controls see text.

by ovariectomy. Following the last day of treatment the reproductive tracts of all experimental and control animals were fixed for histological study.

Results. Examination of table 1 will show that both the acetone and ether fractions contained estrogens. However, in spite of the fact that full estrous smears were taken as early as the day following the third application of extract, the response could not be considered as typical since the uterus remained virtually unaffected. Histologically the vaginal epithelium of all the positively reacting animals showed active growth, the newly grown cells of the epithelial layer standing out sharply as a darkly stained band (eosin and hematoxylin stain). This reaction is illustrated in a previous report.[6]

Since no pronounced estrous reaction could be produced by the subcutaneous route it seems evident that each dosage of extract contained less than 1 standard rat unit of estrogen. However, in the absence of a pure product, the low equivalent of estrogen to ovarian tissue may be more apparent than real. Since the primary object of this study was to determine qualitatively for the estrogenic content of the extracts used, no attempt was made to determine the M.E.D.

Summary. Extracts of the ovaries of the winter flounder were capable of establishing full estrous responses in the vagina of the mature, ovariectomized rat when applied directly to the vagina in daily doses of 0.50 cc. The uterus, however, showed no demonstrable estrous changes indicating that the extracts probably contained less than one standard rat unit per dose.

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